



THE UNIVERSITY *of* EDINBURGH

Title	Chitinase from <i>Vibrio carchariae</i> : purification, gene isolation, and sequence determination
Author	Suginta, Wipa.
Qualification	PhD
Year	1998

Thesis scanned from best copy available: may contain faint or blurred text, and/or cropped or missing pages.

Digitisation Notes:

- Pagination errors in original: page 161 is missing

**A CHITINASE FROM *Vibrio carchariae* : PURIFICATION,
GENE ISOLATION, AND SEQUENCE DETERMINATION**

Wipa Suginta

**Doctor of Philosophy
Department of Biochemistry
University of Edinburgh**

1998



Declaration

The work in this thesis is the result of my own research unless otherwise stated, and has not been presented for any other academic degree elsewhere.

Wipa Suginta

Edinburgh, Nov 1998

CONTENTS

Declaration	I
Contents	II
Dedication	XI
Acknowledgements	XII
List of Figures	XIII
List of Tables	XVII
Abbreviations	XVIII
Abstract	XXIII

CHAPTER 1: Introduction

1.1 General considerations	1
1.2 Classification of glycosyl hydrolases	6
1.3 Chitin utilisation by marine bacteria	13
1.4 Classification of bacteria: <i>Vibrio</i>	16
1.5 Occurrence and roles of chitinases	19
1.6 Mechanism	22
1.6.1 Double displacement mechanism	23
1.6.2 Single displacement mechanism	23
1.6.3 Anchimeric stabilisation hydrolysis mechanism	26
1.7 Structure-function studies on the chitinolytic enzymes	31
1.8 Applications	36
1.8.1 Agriculture	36
1.8.2 Biomedicine	38
1.8.3 Biological conversion process	39
1.9 Literature search for chitinase studies	43
1.10 Aim of Project	44

CHAPTER2: Materials and methods

2.1 Materials	45
2.1.1 Determination of chitinase activity	45
2.1.2 Bacterial strains	45
2.1.3 Bacterial growth	46
2.1.4 Protein purification	46
2.1.5 Carbohydrate Analysis	46
2.1.6 Antibodies production	47
2.1.7 Western blotting	47
2.1.8 Plasmids	47
2.1.9 Oligonucleotides	47
2.1.10 DNA isolation and purification	48
2.1.11 Enzymes for DNA manipulation	48
2.1.12 DNA sequencing	48
2.1.13 Miscellaneous	48
2.2 Methods	50
2.2.1 Development of assay method for chitinase studies	50
2.2.1.1 Determination of chitinase activity by radioactive assay	50
2.2.1.1.1 Preparation of radiolabelled chitin substrate	50
2.2.1.1.2 Measurement of chitinase activity	51
2.2.1.2 Determination of chitinase activity by viscometric assay	51
2.2.1.2.1 Preparation of glycol-chitin	52
2.2.1.2.2 Determination of degree of acetylation of glycol-chitosan	52
2.2.1.2.3 Measurement of chitinase activity	52
2.2.1.3 Determination of chitinase activity by colorimetric assay	53
2.2.1.4 Definition of chitinase activity	53
2.2.2 Expression of chitinases in marine bacteria <i>Vibrio</i>	54

2.2.2.1 Bacterial growth and storage	54
2.2.2.2 Preparation of swollen chitin	54
2.2.2.3 Effect of types of chitin on chitinase expression	55
2.2.2.4 Effect of time course on chitinase expression	55
2.2.2.5 Expression of chitinase from marine <i>Vibrio</i> on chitin plates	55
2.2.3 Attempts to develop a purification scheme for chitinase from <i>V. alginolyticus</i>	55
2.2.3.1 Ammonium sulphate precipitation	55
2.2.3.2 Chromatography on DEAE-Sephadex A-50	56
2.2.3.3 Chitin affinity chromatography	57
2.2.4 Purification of a chitinase from <i>Vibrio carchariae</i>	57
2.2.4.1 Effect of Amount of chitin on chitinase expression	57
2.2.4.2 Effect of concentrations of guanidine HCl on release of chitinase from chitin affinity	58
2.2.4.3 A complete purification of <i>V. carchariae</i>	58
2.2.5 Determination of protein concentration	60
2.2.6 Determination of the subunit molecular weight of chitinase SDS/PAGE	60
2.2.7 Determination of chitinase activity under non-denaturing gel electrophoresis	61
2.2.8 Determination of native molecular mass of chitinase	61
2.2.9 Carbohydrate analysis after chitin affinity chromatography	62
2.2.9.1 Release of carbohydrate from affinity-purified chitinase	62
2.2.9.2 Sample preparation for carbohydrate analysis	62
2.2.9.3 Paper chromatography	63
2.2.9.4 Paper electrophoresis	63
2.2.9.5 Stain for paper chromatograms	64
2.2.9.6 Quantitative analysis	65
2.2.10 Western Blotting	65
2.2.11 Preparation of polyclonal antibodies from the purified <i>V. carchariae</i> chitinase	67

2.2.12 Determination of titre of antiserum	67
2.2.13 Determination of Specificity of antiserum	67
2.2.14 Determination of <i>N</i> -terminal amino acid sequence of <i>V. carchariae</i> chitinase	68
2.2.15 Agarose gel electrophoresis	68
2.2.16 Plasmid DNA preparation	69
2.2.16.1 Small scale preparation	69
2.2.16.2 Large scale preparation of plasmid DNA	70
2.2.17 Bacterial transformation	71
2.2.17.1 Preparation of competent cells	71
2.2.17.2 Introduction of DNA into the competent <i>E. coli</i> cells	73
2.2.18 Polymerase chain reaction for genomic DNA library screening	73
2.2.18.1 Design of oligonucleotide primers	73
2.2.18.2 Isolation of genomic DNA from <i>V. carchariae</i>	74
2.2.18.3 Preparation of DNA template	75
2.2.18.4 Polymerase chain reaction (PCR)	77
2.2.18.5 Analysis and purification of PCR products on gel electrophoresis	77
2.2.18.6 Preparation of DNA template for DNA sequencing	79
2.2.18.7 Sequence analysis of PCR products	79
2.2.19 Preparation of genomic DNA library from <i>Vibrio carchariae</i>	81
2.2.19.1 Preparation of Sau3A I partial digests	81
2.2.19.2 Preparation of pBluescript II KS(-) vector for ligation	81
2.2.19.3 Gene cloning	82
2.2.19.3.1 Small scale ligation	82
2.2.19.3.2 Large scale ligation	83
2.2.20 Screening of genomic DNA library	83
2.2.20.1 Immunological assay	84
2.2.20.2 Chitin plate assay	84
2.2.21 Expression of the clones carrying chitinase gene	84

2.2.22 Restriction investigation of the clones carrying chitinase	85
2.2.22.1 <i>EcoR</i> I Digestion	85
2.2.22.2 Restriction maps of P1C1 and P3C1 clones	85
2.2.23 Determination of DNA sequence of the clone carrying a chitinase gene	86
2.2.23.1 Localisation of the chitinase gene in DNA insert	86
2.2.23.2 DNA sequence of P3C1/ <i>Cla</i> I clone	86
2.2.23.3 Manual sequencing of the upstream end of P3C1 clone	86
2.2.23.4 A complete sequence of the entire chitinase gene	88
2.2.23.5 Computing analysis	88
2.2.24 Preliminary studies of recombinant protein expressed by P3C1 clone	88
2.2.24.1 Protein processing	88
2.2.24.2 Localisation of the recombinant chitinase	90

CHAPTER3: Expression of chitinase in marine bacteria: *Vibrio*

3.1 Introduction	91
3.2 Evaluation of assay methods for chitinase activity	92
3.2.1 Determination of chitinase activity by radioactive assay	92
3.2.2 Determination of chitinase activity by viscometric assay	94
3.2.3 Determination of chitinase activity by colorimetric assay	94
3.3 Expression of chitinases in marine bacteria, <i>Vibrio</i>	97
3.3.1 Effect of time course on chitinase expression	99
3.3.2 Effect of chitin substrates on chitinase expression	101
3.3.3 Expression of chitinase by fourteen species of marine, <i>Vibrio</i>	101
3.3.4 Expression of chitinases on chitin plates	105

3.4 Conclusions and discussion	105
3.4.1 Development of an assay procedure for chitinase studies	105
3.4.2 Expression of chitinase from marine bacteria: <i>Vibrios</i>	107
 <u>CHAPTER4: Purification and characterisation</u>	
4.1 Introduction	110
4.2 Attempts to develop a purification scheme for <i>Vibrio alginolyticus</i> chitinase	111
4.2.1 Ammonium sulphate precipitation	112
4.2.2 Chromatography on DEAE-Sephadex A-50	112
4.2.3 Affinity chromatography on chitin	115
4.3 Purification of <i>V. carchariae</i> chitinase	115
4.3.1 Effect of amount of chitin on chitinase expression from <i>V. carchariae</i>	116
4.3.2 Effect of concentrations of guanidine HCl on release of chitinase from chitin affinity	116
4.3.3 A complete purification of <i>V. carchariae</i> chitinase	119
4.4 Partial <i>N</i> -terminal amino acid sequence of <i>V. carchariae</i> chitinase	127
4.5 Production of polyclonal antibodies against <i>V. carchariae</i> chitinase	127
4.5.1 Determination of titre of anti-chitinase serum	127
4.5.2 Determination of specificity of anti-chitinase serum	129

4.6 Carbohydrate analysis	129
4.7 Conclusions and discussion	134
4.7.1 Attempts to develop a purification scheme for <i>V. alginolyticus</i> chitinase	134
4.7.2 Purification of chitinase from <i>V. carchariae</i>	135
4.7.3 Partial <i>N</i> -terminal amino acid sequence	138
4.7.4 Production of polyclonal antibodies	138
4.7.5 Carbohydrate analysis	139

CHAPTER 5: Development of nucleic acid probes for genomic DNA library

5.1 Introduction	141
5.2 Design of degenerate oligonucleotide primers	142
5.3 Attempted development of nucleic acid probes	142
5.3.1 Analysis of PCR products on agarose gel	145
5.3.2 Manual DNA sequencing	145
5.4 Conclusions and discussion	151

CHAPTER 6: Isolation of the gene encoding chitinase from *Vibrio carchariae*

6.1 Introduction	152
------------------	-----

6.2 Preparation of a genomic DNA library from <i>Vibrio carchariae</i>	156
6.2.1 Preparation of genomic DNA for cloning	156
6.2.2 Purification of <i>Sau</i> 3A I partial digests on agarose gel	162
6.2.3 Ligation and introduction of <i>Sau</i> 3A I partial digests into <i>E. coli</i>	162
6.3 Genomic DNA library screening	165
6.3.1 Immunological assay	167
6.3.2 Chitin plate assay	171
6.4 Restriction analysis of the clones expressing chitinase protein	171
6.4.1 <i>Eco</i> R I Digestion	171
6.4.2 Restriction mappings of P1C1 and P3C1	174
6.5 DNA Sequence analysis of a clone expressing chitinase protein	177
6.5.1 Deletion analysis of a clone carrying chitinase	177
6.5.2 Nucleotide sequence of subclone P3C1/ <i>Cla</i> I	183
6.5.3 A Complete sequence of a gene encoding chitinase	186
6.6 Expression of recombinant chitinase in <i>E. coli</i>	193
6.6.1 Localisation of recombinant chitinase in <i>E. coli</i>	193
6.6.2 Protein processing study	195
6.7 Conclusions and discussion	195
6.7.1 Preparation of genomic DNA library from <i>V. carchariae</i>	195
6.7.2 Isolation of clones carrying a chitinase gene	198
6.7.3 Expression of the chitinase gene in <i>E. coli</i>	199

6.7.4 Restriction analysis of P1C1 and P3C1	200
6.7.5 Sequence analysis of the entire chitinase gene	200
6.7.6 Localisation of the recombinant chitinase in <i>E. coli</i>	201
6.7.7 Attempted processing study	203
 <u>CHAPTER 7: General conclusions and discussion</u>	
7.1 Evaluation of assay methods for chitinase activity	205
7.2 Expression of chitinases in marine bacteria, <i>Vibrio</i>	205
7.3 Purification and characterisation of a chitinase from <i>Vibrio carchariae</i>	207
7.4 Attempted development of nucleic acid probes	210
7.5 Isolation of gene encoding chitinase	211
7.6 Expression of the recombinant chitinase in <i>E. coli</i>	213
7.7 Prediction of the three dimensional structure of <i>V. carchariae</i> chitinase	215
7.8 Plan for future work	225
 Appendix I : References	230
Appendix II : Bacterial media	249
Appendix III : Solutions for protein studies	253
Appendix IV : Methods and solutions for molecular biology work	257
Appendix V : Plasmid maps	261
Appendix VI : Blastx search for chitinase sequences	264
Appendix VII : Literature search	268

**For
my father and mother**

Acknowledgements

Many people have contributed to this thesis. I would like to give special thanks to the following people:

Linda Forthergill-Gilmore for her help and advice throughout the course of this project.

Professor Stephen C Fry for his help and advice on development of the assay method for chitinase activity and on carbohydrate analysis.

Professor Brian Austin and Dr. Peter Robertson, Heriot-Watt University, for kindly providing *Vibrio* stocks, and for all their suggestions and advice during the screening experiment.

Dr. Andrew Cronshaw of the Welmet Protein Characterisation Facility for doing the protein sequencing.

Dr. Peter Estibeiro and Dr. Rory Duncan for training me in all the basic techniques on DNA work, for all the facilities that they kindly provided and also for all their suggestions and useful discussions throughout the DNA work.

Ting-Kuang Niu for being a very good friend and his help on DNA analysis.

Dr. Jacqueline Nairn for training me in basic techniques on DNA work.

Dr. Daniel Rigden for his help on amino acid alignments and for molecular modelling of chitinase.

The Royal Thai Government for financial support throughout the course and by paid leave of absence from Suranaree University of Technology.

Finally, I would like to thank my family and husband, Songkot Dasananda, for their enduring love and support through my studies.

List of Figures

Figure	Description	Page
1.1.	The chitin repeating unit	1
1.2	Illustration of structures of α -chitin (A) and β -chitin (B)	2
1.3	Potential enzymatic routes for the degradation of chitin.	5
1.4	Classification of the bacterial chitinases based on the amino acid sequence feature	12
1.5	A schematic represents utilisation of chitin by chitinolytic bacterium, <i>Vibrio funissii</i>	14
1.6	A schematic of the hydrogen bonds served for a hexaNAG substrate bound to barley chitinase	25
1.7	A scheme of the hydrogen bonds observed for the -1-boat hexaNAG binding mode	27
1.8	The minimum energy structure for the -1-boat hexaNAG conformation	28
1.9	The oxazoline ion and the extended oxocarbenium ion intermediates bound to the chitinase A active site	30
1.10	Ribbon diagram of ChiA in an orientation to show the three domains and the groove of the active site	33
1.11	Comparison of putative catalytic regions in bacterial chitinases	35
1.12	The structure of allosamidin, an diacetylchitobiose analogue	38
1.13	Four-step process concept for chitin bioconversion	41
1.14	Two-step process concept for chitin bioconversion	42
2.1	Illustration of the positions of DNA sequence of P3C1/ <i>Cla</i> I clone where each oligonucleotide was designed	87
2.2	The upstream sequence of the DNA fragment carried by P3C1/ <i>Cla</i> I subclone	89

Figure	Description	Page
3.1	Radioactive assay of <i>Serratia</i> chitinase towards [¹⁴ C]chitin	93
3.2	Determination of degree of acetylation of glycol-chitosan	95
3.3	Viscosimetric assay of <i>Serratia</i> chitinase towards glycol-chitin	96
3.4	Determination of λ_{\max} of azure compound	98
3.5	Effect of incubation times on chitinase expression	100
3.6	Effect of chitin substrates on chitinase expression	102
3.7	Expression of chitinases by fourteen species of marine <i>Vibrio</i>	103
3.8	Expression of chitinases on chitin agar plate	106
4.1	Ion-exchange chromatography on DEAE-Sephadex A-50 of the 35-70% ammonium sulphate precipitate fraction	113
4.2	Effect of amount of chitin on chitinase expression from <i>V. carchariae</i>	117
4.3	Effect of concentrations of guanidine HCl on release of chitinase from chitin affinity	118
4.4	Gel filtration on Sephacryl S200 HR	121
4.5	SDS-PAGE of Superose fplc fractions	123
4.6	Anion-exchange chromatography on Mono Q fplc	124
4.7	SDS-PAGE characterisation of chitinase purification Fractions	125
4.8	Analysis of chitinase activity under non-denaturing electrophoresis conditions	126
4.9	Determination of titre of anti-chitinase serum	128
4.10	Determination of specificity of anti-chitinase serum	130
4.11	Detection of glucosamine in the CA fraction by paper chromatography	131
4.12	Detection of glucosamine in the CA fraction by high-voltage paper electrophoresis	132
4.13	Determination of total carbohydrate content of the CA fraction	133

Figure	Description	Page
5.1	Illustration of two regions of <i>N</i> -terminal amino acids of the <i>V. carchariae</i> chitinase where the primers P1, P2, and P3 were designed.	143
5.2	Illustration of <i>Sau</i> 3A I partial digests that were used as DNA templates in the PCR experiment	144
5.3	Gel electrophoresis of PCR products (P1T3 and P1T7)	146
5.4	Gel electrophoresis of PCR products (P1M13 and P1M13(rev))	147
5.5	Gel electrophoresis of p-GEM-T clones carrying PCR products	148
5.6	A physical map of pGEM-T vector carrying DNA insert from <i>V. carchariae</i>	149
6.1	An agarose gel of <i>Sau</i> 3A I partial digests (I)	159
6.2	An agarose gel of <i>Sau</i> 3A I partial digests (II)	160
6.3	Large-scale preparation of <i>Sau</i> 3A I partial digests	163
6.4	Investigation of transformed <i>E. coli</i> for colonies carrying DNA inserts	166
6.5	Immunological screening of genomic library with anti-chitinase Serum	168
6.6	Western blot analysis of clones carrying DNA insert (I)	169
6.7	Western blot analysis of clones carrying DNA insert (II)	170
6.8	Chitin plate assay of clones carrying chitinase	172
6.9	<i>Eco</i> R I digestion of clone P1C1 and P3C1	173
6.10	Restriction patterns of P1C1 and P3C1 on an agarose gel	175
6.11	Restriction mappings of P1C1 and P3C1	176
6.12	Deletion analysis of P1C1 and P3C1 (I)	178
6.13	Deletion analysis of clone P1C1 (II)	179
6.14	Deletion analysis of clone P3C1 (III)	180
6.15	Chitinase expression of subclones P1C1/ <i>Cla</i> I, P1C1/ <i>Sal</i> I, P3C1/ <i>Cla</i> I, and P3C1/ <i>Sal</i> I	181

Figure	Description	Page
6.16	A nucleotide alignment of the upstream ends of subclone P3C1/ <i>Cla</i> I and the nucleotide sequence of ChiA from <i>Serratia marcescens</i>	184
6.17	Illustration of oligonucleotides designed for determination DNA sequence of subclone P3C1/ <i>Cla</i> I	185
6.18	Nucleotide sequence of subclone of P3C1/ <i>Cla</i> I	187
6.19	Effect of IPTG on chitinase expression of clone P3C1 and subclone P3C1/ <i>Cla</i> I	188
6.20	The entire nucleotide sequence of <i>V. carchariae</i> chitinase	190
6.21	Localisation of the recombinant chitinase from <i>E. coli</i>	194
6.22	Protein processing of the recombinant chitinase in <i>E. coli</i>	196
7.1	Localisation of the chitinase gene in a 4.0-kb DNA insert of the clone P3C1	214
7.2	Multiple alignment of deduced amino acids sequences of some bacterial chitinases	217
7.3	A comparison of amino acid sequence of chitinase between <i>S. marcescens</i> chitinase and <i>V. carchariae</i>	219
7.4	The wire model of chitinase from <i>V. carchariae</i>	222
7.5	The space-filling model of chitinase from <i>V. carchariae</i>	223
7.6	A ribbon structure of chitinase from <i>V. carchariae</i>	224

List of Tables

Table	Description	Page
1.1	Global estimates of annually accessible chitinaceous materials as potential chitin source	4
1.2	Sequence similarity-based classification of glycosyl hydrolases	7-11
1.3	Characteristics that distinguish species of <i>Vibrio</i>	17-18
1.4	Summary of economic analysis of shrimp shell bioconversion process	43
2.1	Optimisation of molar ratio of DNA insert and DNA vector	82
3.1	Determination of <i>S. marcescens</i> chitinase by colorimetric Assay	99
3.2	Chitinase activity obtained from twelve species of <i>Vibrio</i>	104
4.1	Partial purification of chitinase from <i>V. alginolyticus</i> strain ATCC17749	114
4.2	A Complete purification of <i>V. carchariae</i> chitinase	120
6.1	The summary of chitinase studies in marine bacteria	157-158
6.2	Optimisation of genomic DNA insert : pBluescript vector ligation	164
6.3	Signal peptide fragment (1-22) composition analysis	191
6.4	Predicted structural class of the whole and the mature <i>V. carchariae</i> chitinases	192

Abbreviations

A) The amino acids

<u>One letter code</u>	<u>Three letter code</u>	<u>Name</u>
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

B) Proteins

BSA - Bovine serum albumin

ChiA -chitinase A

ChiB - chitinase B

ChiC -chitinase C
ChiN domain - *N*-terminal domain
Chitobiase - *N,N'*-acetylchitobiase
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
 β -GlcNAccidase - *N*-Acetyl- β -glucosaminidase
Endo F1 - Endo- β -*N*-acetylglucosaminidase F1
Endo H - Endo- β -*N*-acetylglucosaminidase H
Fn III - fibronectin type III

C) Micellaneous

A - adenine
Å- angstrom
 A_{260} - absorbance at 280 nm
 A_{280} - absorbance at 260 nm
Ac- - acetyl group
AFLP - amplified fragment length polymorphism
ATP -adenosine-5'-triphosphate
BIDS - Bath Information and Data Services
bp - base pairs
C - cytosine
°C - Degree Celsius
CA -chitin affinity fraction
Chitobiose - *N,N'*-acetylchitobiose
CTAB - cetyltrimethylammonium bromide
cDNA - DNA complementary to RNA
DEAE - diethylaminoethyl
dH₂O - distilled water
DMF - *N,N'*-dimethylformamide
DMSO - dimethylsulfoxide
dNTPs - deoxynucleotide -5'-triphosphate
DNA - deoxyribonucleic acid

DTT - dithiothreitol
 ECL - enhanced chemiluminescence
 ED₅₀ - 50 % lethal dose
 EDTA - ethylenediaminetetra acetic acid
 Fru-6-P - fructose-6-phosphate
 FPLC - fast protein liquid chromatography
 G - guanine
 GlcNAc - *N*-acetylglucosamine
 GlcNAc₂ - *N,N'*-β-D-diacetylchitobioside
 GlcNAc₃ - *N,N'N''*-β-D-triacetylchitotrioside
 GlcNAc-6-P - *N*-acetylglucosamine -6- phosphate
 GlcNH₂-6-P - *N*-acetylglucoamino-6-phosphate
 Guanidine HCl -guanidine hydrochloride
 HEWL - hen egg white lysozyme
 hexaNAG - *N,N',N'',N''',N''''*, hexa-acytylo-chitohexaose
 tetraNAG - *N,N',N'',N'''* tetra-acytylo-chitotetraose
 HPLC - high performance liquid chromatography
 IPTG - isopropyl-β-D-thiogalactopyranoside
 kb - kilobase
 kDa - kilodalton
 K_i - dissociation constant of enzyme-inhibitor complex
 K_m - Michaelis constant
 kV - kilovolt
 λ_{max} - maximum wavelength
 L - litre
 µl - microlitre
 µg - microgram
 M - molar
 mCi - millicurie
 MES - 2-[*N*-morpholino]ethanesulfonic acid
 mg - milligram
 mM millimolar

MIR - multiple isomorphus replacement
 ml - millilitre
 MOPS - 4-morpholinepropanesulfonic acid
 M_r - relative molecular mass
 4-MU-[GlcNAc]₂ - methylumberriferryl-*N,N'*-β-D-diacetylchitobioside
 4-Mu-[GlcNAc]₄ - 4-methylumberriferryl-*N,N',N'',N'''*-tetraacetyl-β-D-chitotetraoside
 NAG - glucosamine
 OD - optical density
 PAGE - polyacrylamide gel electrophoresis
 PAHBAH - p-hydroxybenzoic acid
 PBS-T phosphate buffered saline-tween 20
 PCR - polymerase chain reaction
 PEP: phosphoenol pyruvate
 pI - isoelectric point
 PMSF - phenylmethylsulfonylfluoride
 pK_a - the pH at which an acid is half dissociated
 PST - phosphoenol pyruvate:glucose phosphotransferase system
 PTH - phenylthiohydantoin
 PC - paper chromatography
 PE -paper electrophoresis
 rpm - revolutions per minute
 S200 - Sephacryl S200-HR fraction
 SDS - sodium dodecyl sulphate
 T - thymine
 TCA -trichloroacetic acid
 TEMED - *N,N,N',N'*-tetramethylethylenediamine
 TE - 10 mM Tris. HCl pH 8.0, 1mM EDTA
 TFA - trifluoroacetic acid
 Tris - tris (hydroxymethyl) amino methane
 U - 1 unit of chitinase
 UV - ultraviolet

VCM - Vibrio Complex Medium

v/v - volume by volume

w/v - weight by volume

WGA - wheat germ agglutinin

X-Gal - 5-bromo-4-chloro-3-indolyl- β -D-galactoside

Abstract

Fourteen species of *Vibrio* were screened for chitin-induced chitinase activity in the culture medium. *V. carchariae* and *V. alginolyticus* 283 showed high levels of activity, and screening on agar plates containing swollen chitin showed high expression of chitinase by the same two species, and also by *V. fischeri* and *V. alginolyticus* 284. An affinity purification procedure was developed for the major chitinase from *V. carchariae*. The purified chitinase was active as a monomer with M_r 63000-66000, and displayed activity toward both chitin and 4-methylumbelliferyl-chito-oligosaccharides. *N*-terminal sequence analysis confirmed that the enzyme belongs to the ChiA family of chitinases. The gene encoding chitinase was isolated from a *V. carchariae* genomic library and cloned in pBluescript II KS(-) vector and *Escherichia coli* XL1 Blue. The gene had an open reading frame (2,550 bp) that encodes 850 amino acids. *E. coli* harbouring the vector with a 4.0-kb DNA insert expressed a 95 kDa precursor chitinase which was accumulated in inclusion bodies. The polypeptide sequence of *V. carchariae* chitinase showed 54 %, 53 %, and 52 % identity with ChiA from *Alteromonas* sp. strain O-7, ChiA from *Enterobacter agglomerans*, and ChiA from *Serratia marcescens*, respectively. Homology modelling of the three-dimensional structure of *V. carchariae* chitinase revealed extensive similarity with the known structure of ChiA from *S. marcescens*. The structure comprises three major domains, *N*-terminal domain, catalytic α/β -barrel domain, and $\alpha+\beta$ -small domain. Glu315 and Asp391 located at the active site are suggested to play important roles in the enzyme catalysis.

Chapter 1

Introduction

1.1 General Considerations

Chitin is the most abundant of those polysaccharides that contain amino sugars. Chitin is a linear polymer of β -(1 \rightarrow 4)-linked 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine or GlcNAc) residues (Figure 1.1). Chitin is found in most fungi, mycelial yeasts, green algae, and several species of brown and red algae. It is not present in bacteria, true yeasts, and *Actinomycetes*. Chitin is also widespread in the animal kingdom, occurring in the form of sheets as in the cuticles of arthropods, annelids, and molluscs, or in the form of well-oriented fibres as in the mandibular tendon of lobster (Ward and Seib, 1970).

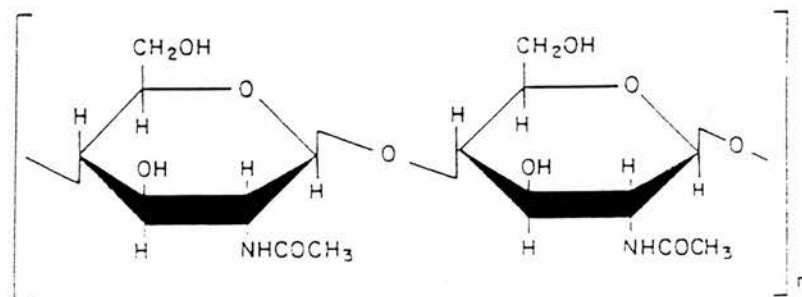
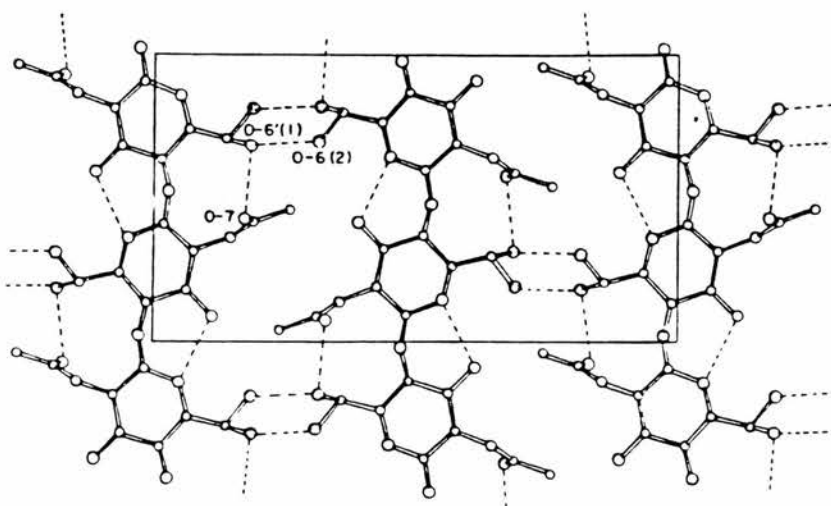


Figure 1.1 The chitin repeating unit.

Chitin seldom occurs alone in nature, one exception being the fibrous chitin produced by the diatom *Thalassiosira fluviatilis*, but generally occurs as the fibrous component of the exoskeletal tissues of many lower animals. The matrix in these composite materials is protein, and this is sometimes extensively calcified. The proportion of chitin in different parts of cuticle varies with the hardness of that part; hardened portions contain smaller proportions of chitin and larger proportions of either inorganic salts (principally calcium carbonate) or cross-linked protein, or both. Chitin in fungi is likewise associated intimately with other materials, and yields of purified chitin from fungi are frequently less than 5% (Ward and Seib, 1970).

The molecular structure of chitin is well established. X-ray diffraction investigations have shown that chitin contains highly ordered regions giving one of several possible crystallographic patterns. *N*-acetylglucosamine residues that forms chitin polymers can be arranged in anti-parallel (α), parallel (β), or mixed (γ) strands, with the α conformation being the most abundant. The illustration of α -chitin and β -chitin structures by X-ray diffraction is shown in Figure 1.2.

A)



B)

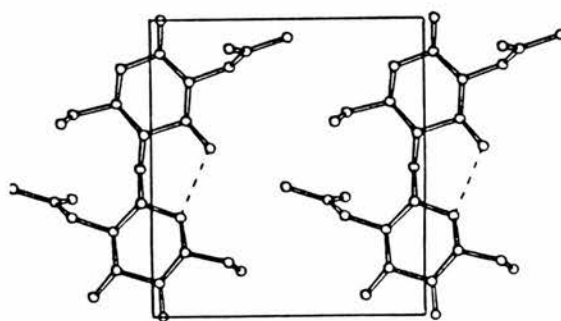


Figure 1.2 Illustration of structures of α -chitin (A) and β -chitin (B) (Blackwell, 1988).

Chitin is insoluble in water, organic solvents, and ammonium reagents but dissolved with some depolymerisation in concentrated mineral acids. Chitin is also soluble in anhydrous formic acid and is dispersed in concentrated aqueous solutions containing certain lithium or calcium salts. Chitin is less reactive than cellulose because of its general insolubility. It is slowly *N*-acetylated and degraded by strong alkali to give a complex mixture of partially deacetylated products collectively termed chitosan. Chitan, completely *N*-deacetylated chitin, is obtained by retreatment of chitosan with alkali or fractionational precipitation of aminopolymer as its hydrochloride salt. Chitin is difficult to acetylate or methylate by methods commonly used to form derivatives of cellulose, since it does not swell in the usual reaction media (Ward and Seib, 1970).

The availability of substantial quantities of chitin has stimulated much research and development in such diversified fields such as medicine, pharmaceuticals, nutrition, agriculture, wastewater treatment, and other commercial applications. Recently, chitin has received attention as a source for exploitation of biomass. It has been reported that multimillion-ton quantities of chitin wastes are released per year by manufacturing processes in the food industry, especially seafood industry, which is creating a serious pollution problem to the nearby environment. Table 1.1 shows recent worldwide estimates of annual chitin bioproduction: it was about 150×10^3 metric tons per annum (Brine, 1984).

The abundance of chitin in both the marine and terrestrial environments has led to an interest in developing processes to modify the structure of chitin and to develop chitin to a utilisable carbohydrate. Current processes use severe chemical reactions that generate unwanted by-products, eliminate potentially important polymers, and reduce chain length. A biological approach that includes the cloning of genes involves in the hydrolysis and modification of chitin could lead to the production of chitin-modifying enzymes in previously unattainable levels and at relatively low cost. Production of these enzymes would allow controlled derivation and modification of chitin to be carried out under extremely mild conditions yielding new and uniform products.

Table 1.1. Global estimates of annually accessible chitinaceous materials as potential chitin source.

Chitin Resources	Quantity landed	Fraction of landing (%)	<u>Chitin-containing waste</u>			Chitin potential
			Wet weight	Solids (%)	Dry weight	
Shellfish	1,700	50-60	468	30-35	154	39
Krill	18,000	40	3,640	22	801	56
Clam/						
Oysters	1,390	65-85	521	90-95	482	22
Squid	660	20-40	99	21	21	1
Fungi	790	100	790	20-26	182	32
Total	22,740		5,118		1,640	150

Estimates are based upon mean values and are given in 10³ metric tons.

The complete enzymatic hydrolysis of chitin to free GlcNAc requires the consecutive action of two hydrolases; chitinase (chitin glycanohydrolase) and *N*-acetylglucosaminidase (*N,N'*-acetylchitobiase) or (chitobiase). These enzymes are often found together in microorganisms and are present in a wide variety of plant as well as animal species. In general, chitinases randomly attack free strands of the chitin polymer, releasing intermediate sized chitin oligosaccharides. Those oligomers are then hydrolysed to the eventual end products *N,N*-diacetylchitobiose (chitobiose) with a small amount of GlcNAc products. Endo-*N*-acetylglucosaminidase then splits chitobiose into two molecules of GlcNAc. On the other hand, exo-*N*-acetylglucosaminidase also hydrolyses glycosidic bonds of chito-oligosaccharides that are generated from chitinases, from their terminal-reducing ends and releases GlcNAc end products.

Alternatively, chitin can be degraded via deacetylation process. There are three enzyme systems, endo-chitosanase, exo-chitobiohydrolase or exo-glucosaminidase, involved in this pathway, and glucosamine (GlcN) is generated as the end product.

A model outlining potential paths for enzymatic degradation of chitin is presented in Figure 1.3 (Davis, 1984).

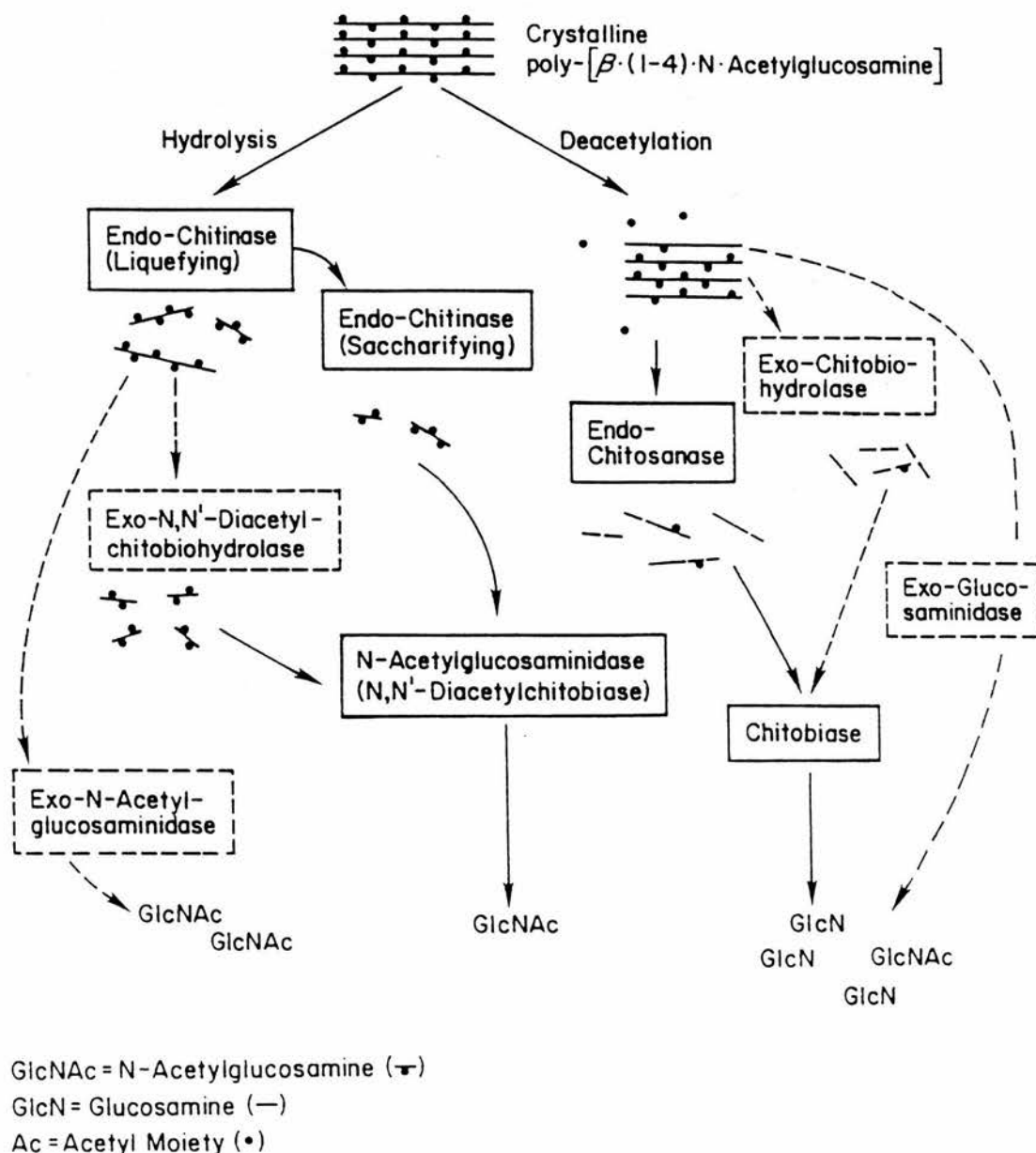


Figure 1.3 Potential enzymatic routes for the degradation of chitin. Confirmed enzyme steps —, hypothetical hydrolysed ---- (Davis and Eveleigh, 1984).

1.2 Classification of Glycosyl Hydrolases

The IUB Enzyme Nomenclature is based on the type of reaction that enzymes catalyse and on their substrate specificity. For glycosyl hydrolases (EC.3.2.1.x), the first three digits indicate the enzyme hydrolysing *O*-glycosyl linkages whereas the last number indicates the substrate and sometimes reflects the molecular mechanism.

This classification is very useful, especially to avoid ambiguities and the proliferation of trivial names, and provides a unique classification. However, such classification is not intended to reflect the structural features of the enzymes. In fact, a classification based primarily on the substrate cannot take into account evolutionary events such as (i) divergence (which can result in changes of specificity, and sometimes reaction type) or (ii) convergent evolution that may force polypeptides with different folds to catalyse the same reaction on the same substrate. Another problem with the EC classification is that it is not appropriate for enzymes showing broad specificity i.e. that act on several substrates.

It is now clear that there is a direct relationship between sequence and folding similarities. Henrissat (1991) has begun a systematic comparison of the primary sequences of glycosyl hydrolases and has found (i) families containing several EC entries and (ii) enzymes with similar substrate-specificities that belong to non-related families. The new classification better reflects sequence (hence structural) similarities and certainly proved to be useful, especially with the rapidly growing number of glycosyl hydrolase genes that are being sequenced and with the concomitant increase in the number of three-dimensional structures being solved. Their classification is based on the comparison of 482 sequences and comprised 45 families with at least two members (Henrissat, 1991; Henrissat and Bairoch, 1993). Almost of the families are polyspecific (contain at least two EC numbers). Only seven sequences have no counterpart and are presently left unclassified. On the basis of amino acid sequence similarities, chitinases have been considered to belong to family 18 and family 19. There is no sequence homology between these families and it has been shown recently that they possess a different mechanism for chitin

cleavage. Family 19 is very homogeneous and contains only plant enzymes, while family 18 is more diverse, and contains enzymes from plants, fungi, and bacteria.

The Henrissat classification of glycosyl hydrolases is summarised in Table 1.2. Moreover, new families in the classification of glycosyl hydrolases (families 46-57) have been added (but the data is not included here) when more than 950 sequences of glycosyl hydrolases have been deposited in databanks (EMBL/GenBank and SWISS-PROT) (Henrissat and Bairoch, 1996).

Table 1.2 Sequence similarity-based classification of glycosyl hydrolases.*

Family	Group of enzymes	EC number
1	β -Galactosidase	3.2.1.23
	β -Glucosidase	3.2.1.21
	6-Phospho- β -galactosidase	3.2.1.85
	6-Phospho- β -glucosidase	3.2.1.86
	Lactase/phlorizin hydrolase	3.2.1.62/108
2	β -Galactosidase	3.2.1.23
	β -Glucuronidase	3.2.1.31
3	β -Glucosidase	3.2.1.21
	Cellodextrinase	3.2.1.74
4	6-Phospho- β -glucosidase	3.2.1.86
	α -Galactosidase	3.2.1.22
5	Endoglucanase	3.2.1.4
	Endoglucanase/cellobiohydrolase	3.2.1.4
	β -Mannosidase	3.2.1.78
	Cellodextrinase	3.2.1.74
6	Endoglucanase	3.2.1.4
	Cellobiohydrolase	3.2.1.91

Classification of glycosyl hydrolases (continued)

Family	Group of enzymes	EC number
7	Endoglucanase	3.2.1.4
	Cellobiohydrolase	3.2.1.91
8	Endoglucanase	3.2.1.4
	Lichinase	3.2.1.73
9	Endoglucanase	3.2.1.4
	Spore-germination specific protein	N.D.**
10	Xylanase	3.2.1.8
	Endoglucanase/cellobiohydrolase	3.2.1.91
	Cellobiohydrolase	3.2.1.91
11	Xylanase	3.2.1.8
12	Endoglucanase	3.2.1.4
13	α -Amylase	3.2.1.1
	α -Glucosidase	3.2.1.20
	Amylase/pullulanase	3.2.1.1/41
	Pullulanase	3.2.1.41
	Cytodextrin gluconotransferase	2.4.1.19
	Maltotetraose-forming amylase	3.2.1.60
	Maltopentaose-forming amylase	3.2.1.-
	Maltohexanose-forming amylase	3.2.1.-
	Isoamylase	3.2.1.68
	Glucodextranase	3.2.1.70
	Neopullulanase	3.2.1.-
	Oligo-1,6- α -glucosidase	3.2.1.10
	Cyclodextrinase	3.2.1.54
14	β -Amylase	3.2.1.2
15	Amyloglucosidase	3.2.1.3
16	Lichinase	3.2.1.73

Classification of glycosyl hydrolases (continued)

Family	Group of enzymes	EC number
16	Laminarinase	3.2.1.39
	Agarase	3.2.1.81
17	Laminarinase	3.2.1.39
	Exo-laminarinase	3.2.1.58
	Lichinase	3.2.1.73
18	Bacterial, fungal, and plant chitinase	3.2.1.14
	Plant chitinase	3.2.1.14/17
	Endo- <i>N</i> -acetyl- β -glucosidase	3.2.1.96
	Endo- <i>N</i> -acetyl- β -glucosidase	3.2.1.96
	Toxin α -chain	3.2.1.14
19	Plant chitinase	3.2.1.14
20	<i>N</i> -Acetyl- β -glucosaminidase	3.2.1.30
	<i>N</i> -Acetyl- β -glucosaminidase	3.2.1.52
21	Lysozyme	3.2.1.17
22	Lysozyme	3.2.1.17
	Lysozyme	3.2.1.52
	Lysozyme	3.2.1.52
23	Lysozyme	3.2.1.17
24	BacteriophageT ₄ lysozyme	3.2.1.17
25	Lysozyme	3.2.1.17
26	β -Mannosidase	3.2.1.78
	Endoglucosidase	N.D.
	Endoglucanase	3.2.1.4
27	α -Galactosidase	3.2.1.22
	<i>N</i> -Acetyl- α -galactosaminidase	3.2.1.49
28	Polygalacturonase	3.2.1.15
	Exo-polygalactoturonase	3.2.1.82

Classification of glycosyl hydrolases (continued)

Family	Group of enzymes	EC number
29	α -Fucosidase	3.2.1.51
30	Glucosylceramidase	3.2.1.45
31	Human α -glucosidase	3.2.1.20
	Sucrase/Isomaltase	3.2.1.48/10
	α -Glucosidase	3.2.1.20
	Amyloglucosidase	3.2.1.3
32	Invertase	3.2.1.26
	Levanase	3.2.1.65
	Levansucrase	2.4.1.10
	Inulinase	3.2.1.7
	Exo- β -fructosidase	3.2.1.80
33	Neuraminidase	3.2.1.18
34	Neuraminidase	3.2.1.18
35	β -Galctosidase	3.2.1.23
36	α -Galactosidase	3.2.1.22
37	Trehalase	3.2.1.28
38	α -Mannosidase	3.2.1.24
39	β -Xylosidase	3.2.1.37
	α -L-Iduronase	3.2.1.76
40	β -Glucosidase	3.2.1.-
41	β -Glucosidase	3.2.1.-
42	β -Galactosidase	3.2.1.23
43	β -Xylosidase	3.2.1.37
	β -Xylosidase/ α -L-arabinofuranosidase	3.2.1.37
44	Endoglucanase	3.2.1.4
	β -Mannosidase/endoglucanase	3.2.1.4

Classification of glycosyl hydrolases (continued)

Family	Group of enzymes	EC number
45	Endoglucanase	3.2.1.4

*This table is modified from Henrissat (1991) and Henrissat and Bairoch (1993).

**N.D. stands for not being identified.

A common observation in most organisms studied so far is the presence of a complex chitinolytic system consisting of several forms of the enzymes. For example, three different types of chitinase are purified and characterised from the medium of cell suspension cultures of barley *Hordeum vulgare* (L.). All of them clearly have differences with regard to molecular size, amino acid composition and sequence (Kragh *et al.*, 1991). Similar results are observed with bacterial chitinases. *Bacillus circulans* WL-12 secretes at least six major chitinases which differ in enzyme activity (Watanabe *et al.*, 1990), more than ten forms of chitinases are produced from *Vibrio harveyi* which are active differently to different types of chitin substrate. They also differ in both their molecular size and recognition by the same anti-chitinase serum (Svityl *et al.*, 1997).

Plant chitinases can be divided into at least three classes based on their amino acid sequence features (Shinshi *et al.*, 1990). Class I chitinases consist of those with a highly conserved main structure and an NH_2 -terminal cysteine-rich domain similar to rubber hevamin. Class II chitinases are structurally homologous to the main structure of class I chitinases but lack the cysteine-rich hevamin domain. Class III chitinases show no homology with either class I or class II chitinases. By comparing amino acid sequences among chitinases and related enzymes, amino acid sequence similarities were found among prokaryotic chitinases, class III plant chitinases, yeast chitinases, endo- β -*N*-acetylglucosaminidase, and yeast killer toxin (Watanabe *et al.*, 1992; Kuranda and Robbins, 1991). Conserved amino acids in the

region sharing amino acid sequence similarities have been suggested to be important for the catalytic activity.

Prokaryotic chitinases are divided into three subgroups based on amino acid features studied by Watanabe *et al.* (1993) (Figure 1. 4). Group A chitinases contain the region (putative catalytic domains) obviously homologous to almost the entire catalytic domain of chitinase A of *B. circulans*. Group B chitinases contain the region homologous to almost the entire catalytic domain of chitinase D of *B. circulans*. Group C chitinase does not show obvious sequence homology with the catalytic domain of either chitinase A1 or chitinase D, except for the regions that are completely conserved in all compared bacterial chitinases.

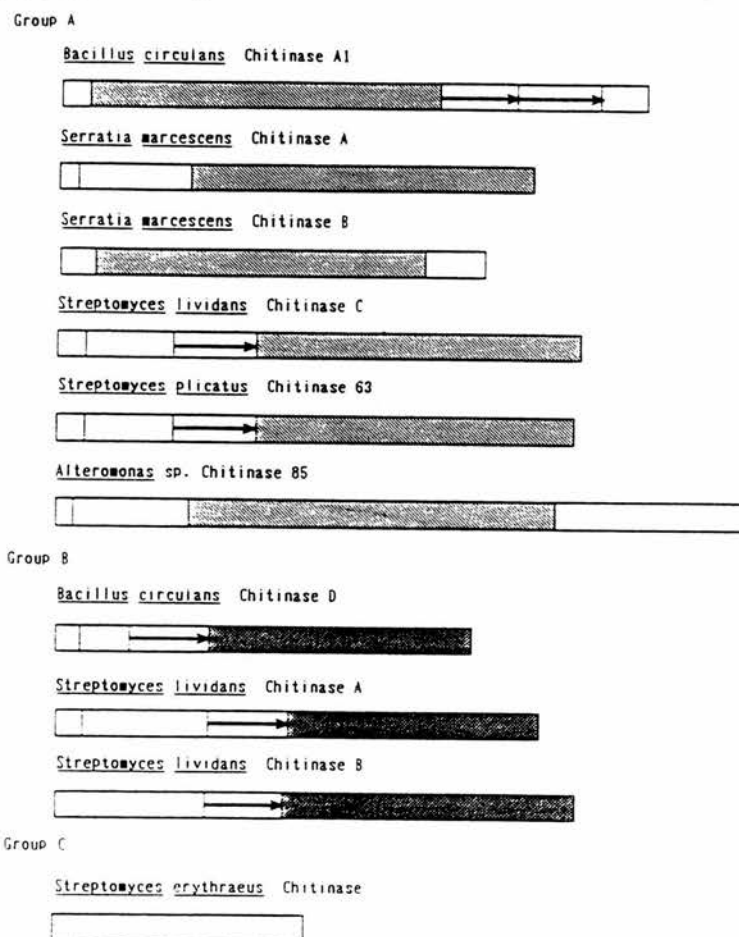


Figure 1.4 Classification of the bacterial chitinases based on the amino acid sequence feature. Shaded boxed indicate the homologous regions of chitinase to the catalytic domain of either chitinase A1 or D of *B. circulans*. Arrow indicates fibronectin type III-like domains (Watanabe *et al.*, 1993).

Based on Watanabe's classification, it is noticed that chitinases from different sources might not be classified into the same group. For example, chitinase A from *Serratia marcescens* was classified in group A while chitinase A from *Streptomyces lividans* was classified in group B, or chitinase B from *S. marcescens* was classified in group A while chitinase B from *S. lividans* was classified in group B. This indicates that nomenclature of the enzymes might not be related to the amino acid sequence similarities.

1.3 Chitin Utilisation by Marine Bacteria

It has been addressed previously that chitin is one of the most abundant organic compounds in nature. The polysaccharide is found in Kingdom fungi (molds and mushrooms), Plantae (green algae), and especially in Animalia (e.g. segmented worms, molluscs, and arthropods).

Chitin is designated "animal cellulose" in the early literature. This explains its wide distribution and great natural abundance. For example, copepods, a single subclass of marine zoo plankton, produce billion of tons of the polysaccharide annually, resulting in a continuous "rain" of chitin on the ocean floor. The ecological significance of this phenomenon was recognised early this decade (Yu *et al.*, 1991; Bassler *et al.*, 1991a; and 1991b). It was pointed out that the ocean would be completely depleted of carbon and nitrogen in a relative short term if chitin could not be returned to the ecological system in a biologically usable form. It was suggested that marine bacteria, such as *Vibrio* spp., play crucial roles in converting these highly insoluble polysaccharides to their carbon and nitrogen sources.

Studies with a marine bacterium *Vibrio furnissii*, suggest a complex chemolytic scheme for chitin utilisation (Figure 1.5). It is suggested that the adhesion of the marine bacteria to chitin-containing particles is a key step of the degradation process. It is shown that the cell must either be able to sense chitin or come into contact with it by random collision. The bacteria must then be able to attach to the polymer, degrade it to soluble oligosaccharides, and these must be further hydrolysed either

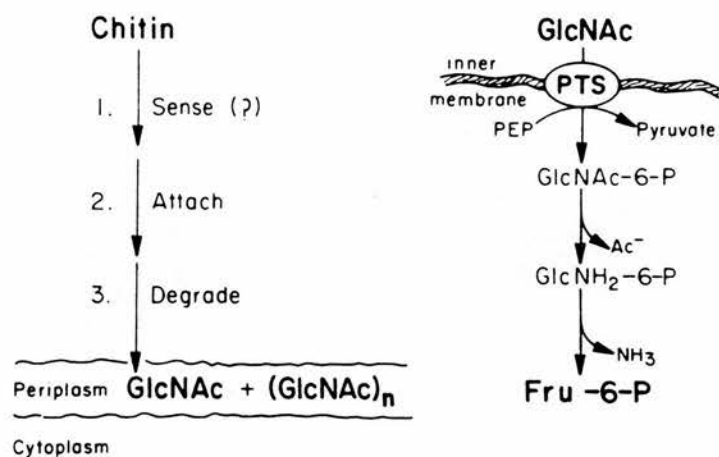


Figure 1.5 A schematic represents utilisation of chitin by chitinolytic bacterium, *Vibrio funissii*. (Yu *et al.*, 1991)

extracellularly or intracellularly to *N*-acetylglucosamine. It has been evidenced that the marine bacterium *V. harveyi* used chitin-binding peptides to mediate the specific attachment to chitin substrate (Montgomery and Kirchman, 1993). This was proved from their study that pre-treatment of chitin particles with wheat germ agglutinin (WGA) and chitinase (purified from *S. marcescens*), which were membrane extracts, inhibited attachment of *V. harveyi* to chitin particulates. The inhibitory effect of WGA and chitinase increased with increasing WGA and chitinase concentrations. This indicates that chitin-binding peptides associated with these membranes are involved in specific attachment. It was suggested that outer membrane-associated chitinases might play a crucial role in specific attachment of the bacterial cells to chitin particles. This was proved from their results that the chitinase-overproducing mutant of *V. harveyi* attached to chitin about twice as much as the wild-type strain. Furthermore, two chitin-binding peptides (40 kDa and 53 kDa peptides) and 150 kDa polypeptides, that are believed to be involved in the specific attachment, cross-reacted strongly to the anti-chitinase serum prepared from the purified 85 kDa chitinase from the mutant of *V. harveyi*.

The chitin degradation processes that are proposed for *V. furnissii* are in principle similar to those given previously, that is, as a combination of enzyme actions (chitinases/chitobiase) except chitodextrinase is suggested to be the key enzyme that is involved in oligosaccharide degradation. In this case, after chitin molecules are degraded by membrane-associated chitinases to oligosaccharide degradation products. The oligosaccharides enter the periplasmic space (possibly via specific porins) where they will be hydrolysed by a unique membrane-bound endoenzyme (chitodextrinase) and an exoenzyme (*N*-acetyl- β -glucosaminidase (β -GlcNAcidase)). The end products in the periplasm, GlcNAc and (GlcNAc)₂ (possibly (GlcNAc)₃) are catabolised by two possible pathways; (i) Disaccharide pathway, a (GlcNAc)₂ permease is apparently expressed by *Vibrio furnissii*. Translocated (GlcNAc)₂ is rapidly hydrolysed by a soluble, cytosolic β -GlcNAcidase, and the GlcNAc is phosphorylated by an ATP-dependent, constitutive kinase to GlcNAc-6-P; (ii) Monosaccharide pathway, periplasmic GlcNAc is taken up by enzyme II (NAG) of the phosphoenolpyruvate:glucose phosphotransferase system, yielding GlcNAc-6-P, the common intermediate for both pathways. Finally, GlcNAc-6-P is inverted to Ac- + GlcNH₂-6-P and then to Fru-6-P + NH₃.

It has been described in a previous report that the periplasmic and cytosolic β -GlcNAcidases were induced differentially by *N,N'*-chitobiose (GlcNAc)₂, suggesting that (GlcNAc)₂ probably serves as the "true" inducer of the chitin degradative enzymes (Bassler *et al.*, 1991). This hypothesis was also strongly supported by a previous work (Soto-Gil and Zyskind, 1984). They reported that chitobiose is a strong inducer of both chitinase and chitobiase secreted by *V. harveyi*. They found that the activity of both enzymes was detected within minutes after the addition of chitobiose in the growth medium. They suggested that chitobiose is rapidly hydrolysed to GlcNAc and almost completely metabolised within one hour after induction even when the additional carbon source, glycerol, was present. It was also concluded from their study that concomitant with the appearance of chitobiose was the reduction in specific activity of chitinase, suggesting very stringent control or possible catabolite repression. However, the mechanism of chitin induction on chitinase and chitobiase at the DNA level still remains inclusive.

1.4 Classification of Bacteria: *Vibrio*

As mentioned previously, bacteria from the family *Vibrionaceae* especially marine *Vibrio* species are a potential source of chitinases. Study of chitinases from marine *Vibrio* has been increasing dramatically over the last decade. Most of the study has been directed towards bioconversion applications in order to develop biochemical approaches for chitin use. Therefore, it would be worthwhile addressing classification of bacteria *Vibrio* in this study.

The *Vibrios* have been characterised as gram-negative, flagellated, motile, facultative anaerobic rod-shaped organisms. They are closely related to bacteria in the family *Enterobacteriaceae*, which includes *Escherichia coli* and *Salmonella typhimurium*. Characteristics that distinguish different species of *Vibrio* are shown in Table 1.3

Vibrio species in Table 1.3 have been classified on the basis of morphotypic i.e. number of polar flagella and biochemical characteristics (ability to produce some particular hydrolases, ability to utilise D-cellulose and citrate, to grow at 35°C, and to reduce nitrogen). It has been noticed that seven *Vibrio* species including *V. alginolyticus*, *V. campbellii*, *V. carchariae*, *V. harveyi*, *V. parahaemolyticus*, *V. pelagius* strain biovar 2, and *V. vulnificus* show similarities in all the characteristics except that they have differences in utilisation of D-cellulose or citrate. This might indicate that all these *Vibrios* are closely related.

A report on classification of 94 *Vibrio* isolates that are closely related to *V. harveyi* was published recently (Pedersen *et al.*, 1998). It was reported that all the isolates were recognised into nine clusters based on amplified fragment length polymorphism (AFLP). The largest cluster was considered to be the bona fide *V. harveyi* group, which contained the type strains of *V. harveyi*, *V. carchariae*, and most of the strains isolated from fish. It was found that *V. harveyi* and *V. carchariae* had high similarities in both DNA-rRNA hybridisation (ribotyping) and DNA-DNA hybridisation patterns. The *V. campbellii* type strain also had several ribotypes in

Table 1.3 Characteristics that distinguish species of *Vibrio* (Ishimaru *et al.*, 1996).

Taxon	Thornley dihydrolase activity	arginine reduction	Nitrate reduction	Chitinase activity	Gelatinase activity	Lipase activity	D-Cellobiose Utilisation of	Citrate	Growth at 37°C	3-12 Polar flagella
<i>Vibrio aestuarianus</i>	+	ND	ND	ND	-	ND	ND	+	+	-
<i>Vibrio alginolyticus</i>	-	+	+	+	+	+	-	+	+	-
<i>Vibrio anguillarum</i>	+	+	+	+	+	+	+	+	+	-
<i>Vibrio campbellii</i>	-	+	+	+	+	+	V	V	+	-
<i>Vibrio carchariae</i>	-	+	+	+	+	+	ND	-	+	-
<i>Vibrio cholerae</i>	-	+	+	+	+	+	ND	V	+	-
<i>Vibrio cincinnatiensis</i>	-	+	+	+	-	+	-	+	+	-
<i>Vibrio costicola</i>	V	V	V	-	-	-	+	+	+	-
<i>Vibrio damsela</i>	+	+	+	+	-	-	-	-	+	-
<i>Vibrio diazotrophicus</i>	+	+	+	-	-	-	+	+	+	ND
<i>Vibrio fischeri</i>	-	V	V	V	-	-	+	+	+	-
<i>Vibrio fluvialis</i>	+	+	+	+	-	+	+	V	V	+
<i>Vibrio furnisii</i>	+	+	+	+	+	+	V	+	+	-
<i>Vibrio gazogenes</i>	-	-	-	+	+	+	-	+	+	ND
<i>Vibrio harveyi</i>	-	+	+	+	+	+	+	+	+	-
<i>Vibrio hollisae</i>	-	+	+	ND	-	-	ND	+	+	-
<i>Vibrio ichthyenteri</i>	-	+	+	-	-	-	ND	-	+	-
<i>Vibrio ilipiscarius</i>	+	+	+	ND	-	ND	-	-	-	-
<i>Vibrio logei</i>	-	+	+	+	-	V	+	-	-	ND
<i>Vibrio marinus</i>	-	+	+	+	+	+	+	-	-	+
<i>Vibrio mediterranei</i>	+	+	+	+	-	+	+	+	+	-
<i>Vibrio metschnikovii</i>	+	-	-	+	-	+	+	ND	ND	-
<i>Vibrio minicus</i>	-	+	+	ND	ND	-	ND	V	+	-
<i>Vibrio mytili</i>	+	+	+	ND	ND	+	+	V	+	-
<i>Vibrio natrigens</i>	-	+	+	-	V	+	V	+	+	-

Characteristics that distinguish species of *Vibrio* (continued)

Taxon	Thornley arginine dihydrolase activity	Nitrate reduction	Chitinase activity	Gelatinase activity	Lipase activity	Utilisation of D-Cellobiose	Citrate	Growth at 37°C	3-12 Polar Flagella
<i>Vibrio navarrensis</i>	-	+	ND	+	ND	V	V	+	+
<i>Vibrio nereis</i>	+	+	V	V	-	-	+	+	-
<i>Vibrio nigripulchritudo</i>	-	+	+	+	+	+	+	-	-
<i>Vibrio ordalii</i>	-	-	V	+	-	-	+	-	-
<i>Vibrio orientalis</i>	V	+	+	+	+	+	+	-	-
<i>Vibrio parahaemolyticus</i>	-	+	+	+	+	-	+	+	-
<i>Vibrio pelagius</i> biovar 1	-	+	V	-	+	-	+	+	-
<i>Vibrio pelagius</i> biovar 2	-	+	+	+	+	-	+	+	-
<i>Vibrio penaeicida</i>	-	+	+	+	+	-	V	+	-
<i>Vibrio proteolyticus</i>	+	+	+	+	+	-	V	+	-
<i>Vibrio salmonicida</i>	-	-	-	-	-	-	ND	+	+
<i>Vibrio species</i> INFL-2	-	+	-	-	-	-	-	-	-
<i>Vibrio splendidus</i> biovar 1	V	+	+	+	+	+	+	V	-
<i>Vibrio splendidus</i> biovar 2	V	+	+	+	+	V	+	-	-
<i>Vibrio tubiashii</i>	+	+	+	+	+	+	+	V	-
<i>Vibrio vulnificus</i> biovar 1	-	+	+	+	+	+	+	+	-
<i>Vibrio vulnificus</i> biovar 2	-	+	ND	+	+	ND	+	+	-

+, Positive in at least 90% of the strains; -, Negative in at least 90% of the strains; V, Variable among strains
ND, No data available.

common with the type strains of both *V. harveyi* and *V. carchariae*, but it showed relatively low similarities in DNA-DNA hybridisation patterns with *V. harveyi*. There was clear distinction between the other species and these three species. Because of high similarities with *V. harveyi* at the DNA level, *V. carchariae* was thereby suggested to be the junior synonym of *V. harveyi*.

However, it was realised in this study that *V. harveyi* appeared to differ from *V. carchariae* in some phenotypic properties, especially in chitinase production. For example, *V. harveyi* contains more than ten different chitinases (Svitil *et al.*, 1997) while *V. carchariae* apparently contains a single major chitinase enzyme. Moreover, immunological data showed that there was no relationship between chitinases secreted from the two *Vibrios* (as indicated by no cross-reaction of *V. harveyi* chitinases with anti-chitinase serum raised against purified *V. carchariae* chitinase). No similarity in the amino acid sequences of ChiA from *V. harveyi* and ChiA from *V. carchariae* was also shown in this study (as discussed in chapter 4). Because of the reasons given above, we still leave *V. harveyi* and *V. carchariae* as different type species.

1.5 Occurrence and Roles of Chitinases

Chitinase (EC.3.2.1.14) is an essential component of chitinolytic systems catalysing the degradation of insoluble chitin. Chitinases have been detected in a variety of organisms, not only in those containing chitin as their major component, such as fungi, and invertebrate species, especially insects and crustaceans, but also in ones which do not contain chitin, such as bacteria, higher plants, and some invertebrate species. The roles of chitinases in these organisms are diverse and subject to intensive investigation. In fungi, chitinases seem to play a morphogenic role during apical growth, cell division and differentiation, as well as a nutritional role related to those species saprophytic and mycoparasitic in fungi (Papavizas, 1985; Cabib, 1987; Kuranda and Robbins, 1991). Chitinases secreted by hyperparasitic fungi seem to be involved in parasitic attacks on various host fungi (Srivastava *et al.*, 1985, Sivan and Chet, 1989).

Immunological and cytochemical studies (Manocha and Zhonghua, 1997) showed that chitinase is found in cytoplasmic vacuoles of an uninfected susceptible fungal host (*Choanephora cucurbitarum*), whereas in the resistant host (*Phascolomyces articulatus*) chitinase was found in the cell wall, as well as in vacuoles. Infection by a biotrophic mycoparasite, *Piptocephalis virginiana*, destroyed vacuolar integrity and chitinase is spread into the cytoplasm in both hosts. Only in a resistant host, is the accumulation of chitinase observed at the penetration site that results in the subsequent development of a papilla, considered as a probable cause of resistance in this host-mycoparasite system. The cell wall-localised chitinase in the resistant host is suggested probably to be involved in recognition processes activating the defences of the host fungus (Mauch and Staehelin, 1989).

In soil borne fungi, species of the genus *Trichoderma* have been described as the best known biological control agent against fungal plant pathogens (Papavizas, 1985). The degradation and further assimilation of the phytopathogenic fungi, namely mycoparasitism, has been proposed as the major mechanism accounting for their antagonistic action. Chitinases and β -1,3-glucanases have been suggested as the key enzymes involved in this process (Cherif and Benhamou, 1990; Elad *et al.*, 1982; and Ridout *et al.*, 1986).

In some other invertebrate species, chitinases are usually part of the digestive tract (Jeuniaux, 1966). In the result of a survey of digestive chitinases in eight species of vertebrates, Jeuniaux (1961) reported the presence of chitinase in gold fish *Carassius auratus* (L.). In subsequent work on the Japanese sea bass, *Lateolabrax japonicus* (Okutani, 1967 and 1977), a comparison of chitinolytic activity in sections of the digestive tract showed that the highest enzyme levels were in the stomach. Similarly, gastric chitinolytic activity was shown to be high in rainbow trout (Okutani *et al.*, 1967). Chitinolytic activity has been studied in the digestive tract of Dover sole *Solea solea* (L.) (Clark *et al.*, 1988). It was found that the principle hydrolysis of chitin takes place in the stomach at pH 2-3 and that chitin oligosaccharides were hydrolysed further down the alimentary canal at pH 5-6. The alkaline chitinolytic activity at pH 8-9 is suggested to be probably of microbial origin. Since the natural

diet of adult Dover sole contains invertebrate crustaceans and molluscs, it is possible that the chitinolytic activity in digestive tract is possibly involved in substrate degradation and uptake processes of the fish. On the other hand, chitinases are associated with the need for partial degradation of old cuticle in insects and crustaceans, and their secretion is controlled by a complex hormonal mechanism (Spindler-Barth *et al.*, 1993).

In plants, chitinases have mostly been investigated in leaf tissues. For example, in cucumber leaf tissue, almost all the chitinase activity seems to be present in the intercellular fluid (Boller and Matraux, 1988), in bean leaf tissue, all the chitinase activity is present in the vacuoles (Mauch and Staehelin, 1989). In tobacco leaf, acidic chitinases are present in the intercellular fluid, whereas the basic chitinases are present in the vacuoles (Keefe *et al.*, 1990). Chitinases are considered to be host-synthesised proteins induced in response to a fungal or insect attack and to contribute to self-defence systems. Chitinases may destroy the cell wall of pathogens by lysis, and the digested fragments may induce other self-defence systems (Mauch *et al.*, 1984; Kurosaki *et al.*, 1987; and Kurosaki *et al.*, 1988).

It has been reported that an ethylene-induced endochitinase in primary leaves of *Phaseolus vulgaris* (L.) has 30-fold higher chitinase activity than the non-treated chitinase, and attacks chitin in isolated cell walls of *Fusarium solani*. It also acts as a lysozyme when incubated with *Micrococcus lysodeikticus*, suggesting a potential defence capacity of the plant cells against pathogens (Boller *et al.*, 1983). In addition to ethylene, it was also suggested from later studies that fungal elicitor or pathogen attacks caused a remarkable activation of chitinase production by increased transcription.

It was discovered that elicitor stimulated a 10-fold higher transcription after 5 minutes and 30-fold increase with 20 minutes. There was a transient accumulation of chitinase transcripts, with maximum levels 2 hours after elicitor treatment, concomitant with a phase of rapid enzyme synthesis. Chitinase transcripts also notably accumulated in wounded and infected hypocotyls. It was indicated from

chitinase cDNA sequences that hybridised to several genomic fragments that chitinase synthesis is a result of several genes in the plant genome (Hedrick *et al.*, 1988).

Moreover, a study conducted with a carrot cell culture of the temperature-sensitive mutant (ts11), which does not proceed beyond the globular stage at the non permissive temperature, suggested that apart from the postulated role in the plant defence response, chitinases also show a function in somatic embryogenesis. In their study, it was found that the addition of the 32 kDa endochitinase to the ts11 embryo cultures at the non-permissive temperature promoted the formation of a correctly formed embryo protoderm. This implies that the enzyme has an important function in the early stage of somatic cell development (de Jong *et al.*, 1992).

In bacteria, endophytic bacteria seem to be important for fungal defence mechanisms for their residing hosts. For example, *Bacillus cereus* strain 65 that is isolated as an endophyte of mustard (*Sinapsis*) plants reduces the incidence of root rot disease caused by *Rhizoctonia solani* when it is inoculated into cotton seedlings (Pleban *et al.*, 1997).

1.6 Mechanism

On the basis of amino acid sequences, the chitinases from families 18 and 19, are unrelated, and differ in structure and mechanism. Sequence analyses show very low similarity between these classes of chitinases. Family 19 chitinases share the bilobal $\alpha+\beta$ folding motif of lysozyme, which forms a well-defined substrate binding clef between the lobes. In contrast, family 18 chitinases shared sequence, which form the catalytic $(\beta/\alpha)_8$ -barrel active site (see Figure 1.10 section 1.7).

The mechanisms for enzyme catalysis that are proposed in this section have been studied based on molecular dynamic simulations. This is due to difficulties in obtaining the crystal structure of the enzymes with the real substrate for mechanism study.

In principle, there are two general mechanistic pathways for acid-catalysed glycosyl hydrolysis which result in the following: (i) retention of stereochemistry of the anomeric oxygen at C1' relative to the initial configuration or (ii) inversion of the stereochemistry.

1.6.1 Double Displacement Mechanism

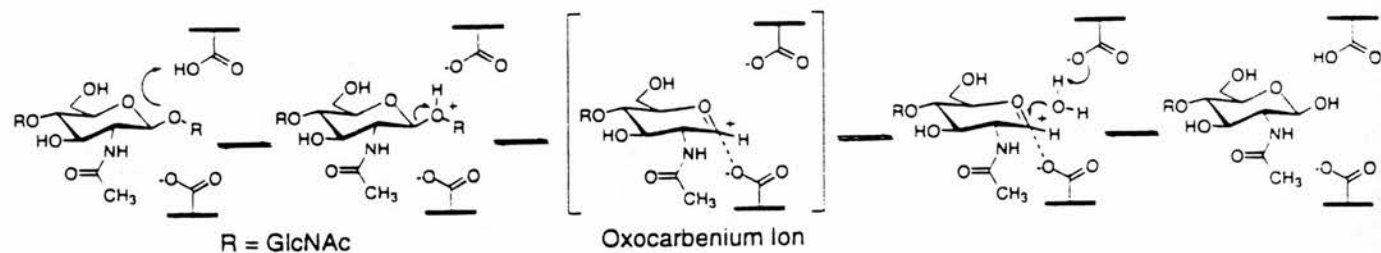
An example of the retaining mechanism is hen egg white lysozyme (HEWL), which has been shown to require two acidic amino acid residues, one of which is protonated. This mechanism is believed to proceed by the initial protonation of β -(1,4)-glycosidic oxygen (leading to an oxocarbenium ion intermediate) and is stabilised by a second carboxylate (either through covalent or electrostatic interactions). Nucleophilic attack by water yields the hydrolysis product, which necessarily retains the initial anomeric configuration. This is commonly referred as the double displacement mechanism of hydrolysis (see Scheme 1)

1.6.2 Single Displacement Mechanism

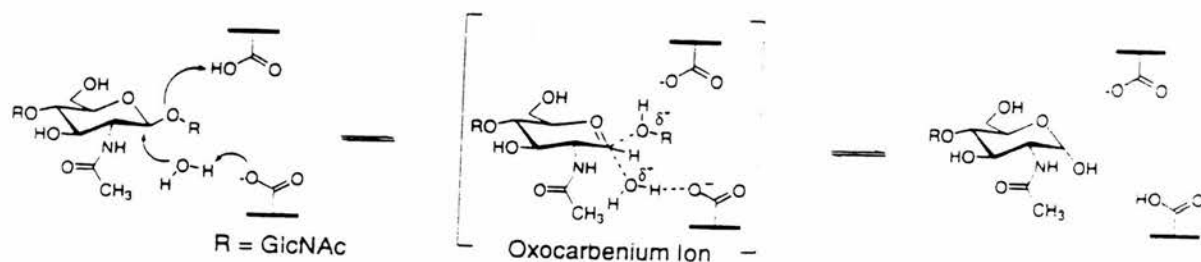
Although the X-ray crystal structure of a family 19 barley chitinase reveals a lysozyme like fold (suggesting a double displacement mechanism), the hydrolysis products for two family 19 chitinases show inversion of the anomeric configuration (Fukamizo *et al.*, 1995; and Iseli *et al.*, 1996). This leads to the second commonly discussed hydrolysis mechanism: a concerted displacement reaction in which a bound water molecule acts as a nucleophile (see Scheme 2). The crystal structure suggests that the second catalytic carboxylate may be sufficiently close to allow co-ordination of a water molecule consistent with a single displacement mechanism.

By using molecular dynamic simulations, Brameld and Goddard (1998a) have examined the binding of hexaNAG substrate and two potential hydrolysis intermediates (an oxazoline ion and an oxocarbenium ion) to a family 19-barley chitinase. They find that the hexaNAG substrate binds with all sugars in a chair conformation, unlike the family 18 chitinases that cause substrate distortion. Glu 67

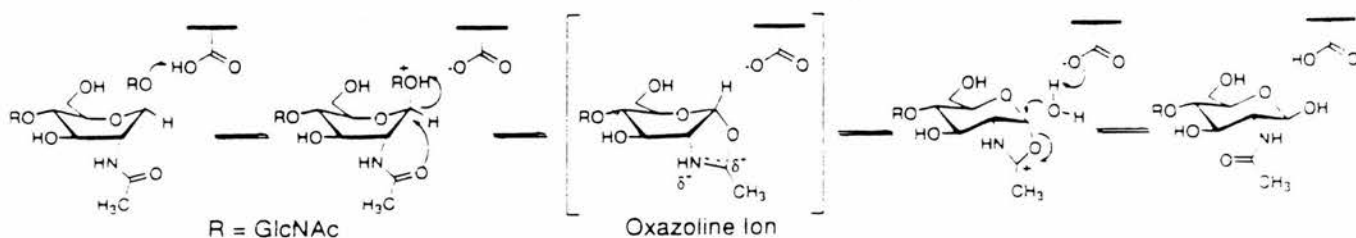
Scheme 1 Double-displacement hydrolysis mechanism, which requires two acidic residues in the active site and leads to retention of the anomeric configuration.



Scheme 2 Single-displacement mechanism hydrolysis mechanism of family 19 chitinases, which requires only one acidic residue in the active site and results in inversion of the anomeric configuration.



Scheme 3 Anchimeric stabilisation hydrolysis mechanism of family 18 chitinases where the substrate is distorted to a boat confirmation and the oxazoline ion intermediate is stabilised through anchimeric assistance from neighbouring C2' N-acetyl group.



is in a position to protonate the anomeric oxygen linking sugar residues D and E whereas Asn 199 serves to hydrogen bond with the C2' *N*-acetyl group of sugar D, thus preventing the formation of an oxazoline ion intermediate (see Figure 1.6). In addition, Glu89, which is suggested to be a part of a flexible loop region, allows a conformational change to occur within the active site to bring the oxocarbonium ion intermediate and Glu89 closer (by 4-5 Å). A hydrolysis product with inversion of the anomeric configuration is suggested as a result of nucleophilic attack by a water molecule that is coordinated by Glu89 and Ser120 (see Schematic 2).

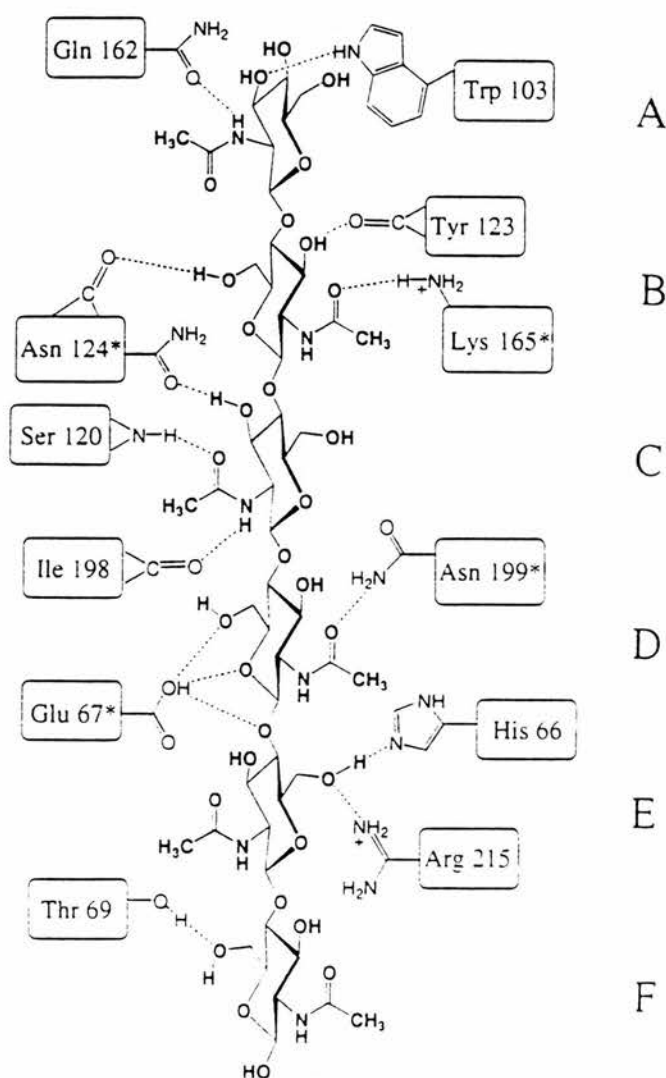


Figure 1.6 A schematic of the hydrogen bonds served for a hexaNAG substrate bound to barley chitinase. *. Residues strongly conserved in family 19 chitinases (Brameld and Goddard, 1998a)

1.6.3 Anchimeric Stabilisation Hydrolysis Mechanism

Family 18 chitinases have been reported to yield hydrolysis products, which retain the anomeric configuration at C1'. However, the X-ray crystal structures of *Serratia marcescens* ChiA (Perrakis, 1994) and hevamine chitinase (van Scheltinga *et al.*, 1994) reveal no second acidic residue in the active site capable of stabilising the oxocarbenium ion. Thus, neither the single nor double displacement mechanism is consistent with the observed structure and hydrolysis products. An increasing body of experimental and theoretical evidence points to an oxazoline ion intermediate formed through anchimeric assistance by the neighbouring *N*-acetyl group (see Scheme 3) as being the likely mechanism for family 18 chitinases.

Using molecular dynamic simulations, the plausible conformations for a hexaNAG substrate bound to the active site of Chi A is examined (Figure 1.7). It is found that (i) the hydrolysis mechanism of ChiA (a family 18 chitinase from *Serratia marcescens*) involves substrate distortion, (ii) the first step of acid-catalysed hydrolysis (protonation of the linking anomeric oxygen between GlcNAc residues -1 and +1) requires a boat conformation for the GlcNAc residue at binding subsite -1 (Figure 1.8); (iii) It is predicted from *ab initio* quantum mechanical calculations (HF/6-31G**) that protonation of a GlcNAc in a boat conformation leads to spontaneous anomeric bond cleavage to yield an oxazoline ion intermediate.

Several conformations of two possible hydrolysis intermediates: the oxazoline ion (Figure 1.9a) and the oxocarbenium ion (Figure 1.9b), have been also studied based on simulations of the bound intermediates with the equilibrated hexaNAG. It was found that only the oxazoline ion orients in the enzyme active site so as to allow stereoselective attack by water. This leads to retention of configuration in the anomeric product as observed experimentally. It is suggested that all family 18 chitinases possibly share a common mechanism. Hence, it is suspected that distortion of the substrate into a boat form of subsite -1 is required for any glycosyl hydrolase which has only one acidic residue in the active site.

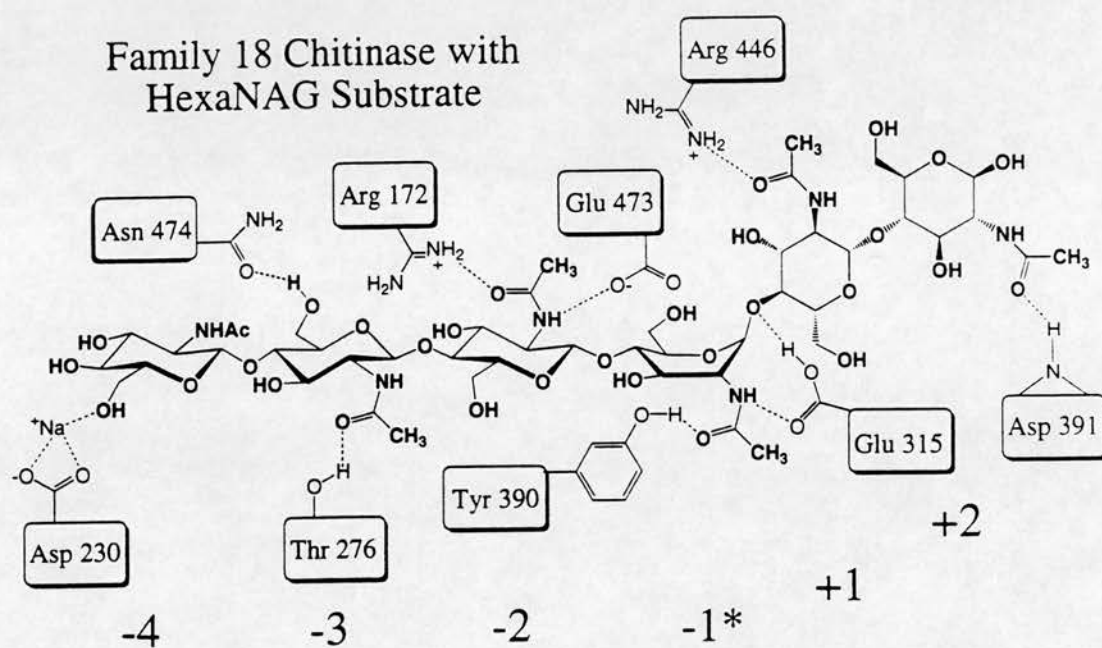


Figure 1.7 A scheme of the hydrogen bonds observed for the -1-boat hexaNAG binding mode. Note: In this schematic representation Tyr 390 appears to the left of the *N*-acetyl carbonyl oxygen of residue -1. However, the three-dimensional representation shows that structure has this carbonyl group is rotated below the plane of the sugar with Tyr 390 positioned on the opposite side of the binding cleft. Thus, the hydrogen bond to Tyr 390 helps to position the *N*-acetyl group prior to formation of an oxazoline ion and will not slow catalysis (Brameld and Goddard, 1998b).

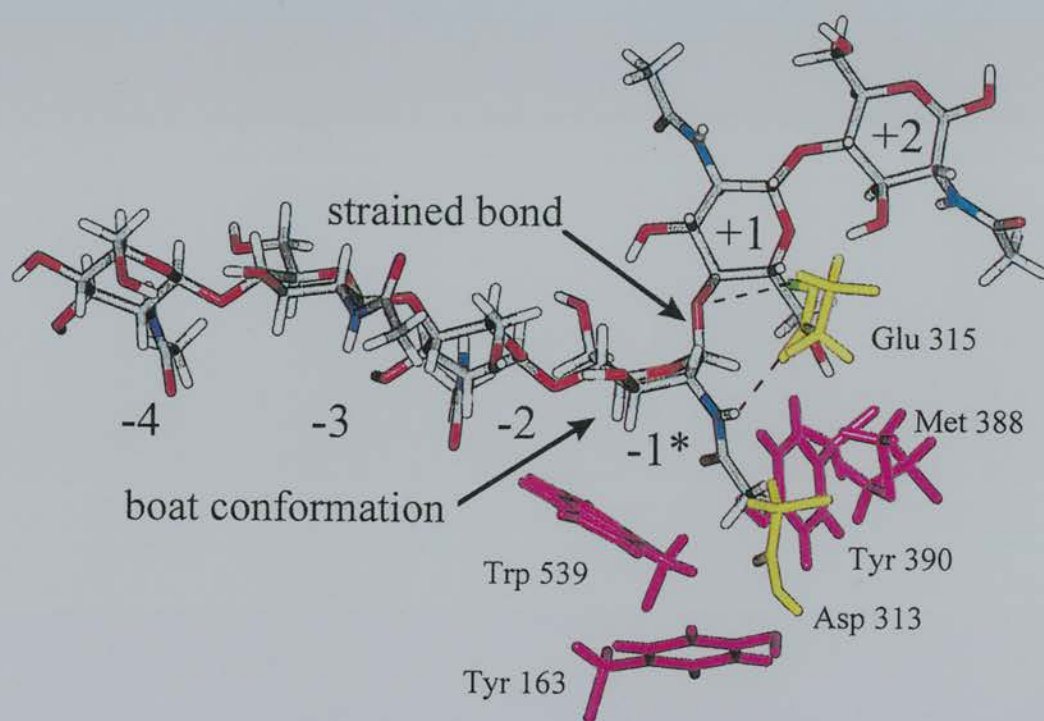


Figure 1.8 The minimum energy structure for the -1-boat hexaNAG conformation. A boat geometry for GlcNAc residue -1 and the twist between residues -1 and +1 strain the linking glycosidic bond are observed. Glu 315 is found to be oriented so as to allow rapid proton transfer to the linking anomeric oxygen and to form a hydrogen bond with the *N*-acetyl amide (Brameld and Goddard, 1998b).

A schematic in Figure 1.8 shows that the -1-boat binding geometry is stabilised through a series of specific hydrogen bonds between each GlcNAc residue and the binding site of the enzyme. "The O6' of sugar -4 interacts with a counter ion bound to Asp 230. The *N*-acetyl carbonyl of sugar -3 forms a hydrogen bond to the side chain of Thr 276 while the O6' hydrogen bonds to Asn 474. Hydrophobic contacts are also made with Trp 167. The *N*-acetamide of sugar -2 donates a hydrogen bond to Glu 473 while the carbonyl accepts a hydrogen bond from Arg 172. Non-specific contacts are made with the hydrophobic "floor" of the binding cleft. Sugar -1 is tightly bound through a hydrogen bond from Tyr 390 to the *N*-acetyl carbonyl and from the acetyl amide to Glu 315 (one oxygen is protonated). There are also critical hydrophobic interactions with Tyr 163 and Tyr 539, which force the -1 sugar into a boat conformation. Sugar +1 is less tightly bound and forms a hydrogen bond between Arg 446 and the *N*-acetyl carbonyl. Similarly, sugar +2 also makes few specific contacts except for a hydrogen between the *N*-acetyl carbonyl and main chain amide hydrogen of Asp 391" (quoted from Brameld and Goddard, 1998b)

It is generally accepted that the first step of the acid-catalysed hydrolysis mechanism of chitinase A involves a proton transfer from Glu 315. Evidence in support of this includes the observation that Glu 315 is completely conserved in family 18 chitinases. In addition, site-directed mutagenesis of the corresponding Glu residue in the *Bacillus circulans* chitinase to a Gln was reported to essentially eliminate activity (Watanabe *et al.*, 1993).

For the chitinase system, the likelihood of proton transfer primarily depends on the distance between the proton donor and acceptor. Making the assumption that Glu 315 is the donor, the proposed proton acceptor is the β -(1,4)-glycosidic oxygen between sugar residues -1 and +1. A plot of the proton-oxygen distance for the hexaNAG simulations showed that the extended *N*-acetyl geometry of the -1-boat conformation places the proton from the glycosidic oxygen is close enough for a direct hydrogen bond is made, while the -1-chair conformation places the glycosidic oxygen too far from Glu 315 and would not lead the rapid hydrolysis. It is concluded that tight

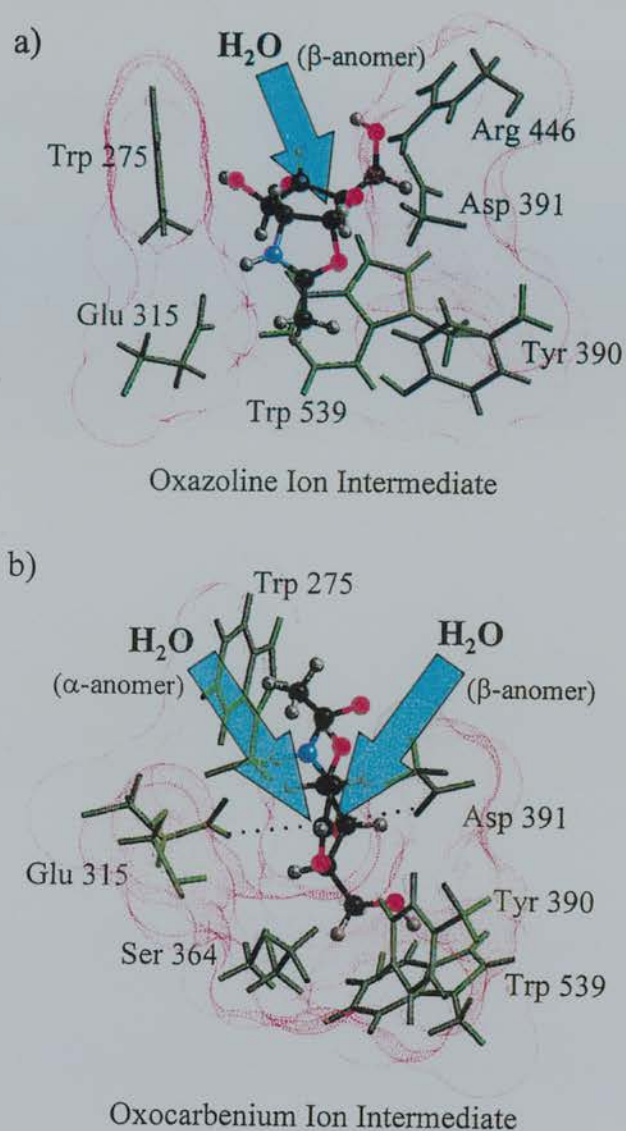


Figure 1.9 (a) The oxazoline ion intermediate bound to the chitinase A active site. Only one face of the oxozaline is open to attack by water at C1' (as indicated with arrow) which lead to a single anomeric product. (b) The extended oxocarbenium ion intermediate is stabilised by Glu315 and Asp 391 (dotted line), and the active site cleft is more narrow than for the oxozaline ion. Attack by water at C1' is hindered equally on both sides of the cleft (as indicated by two arrows) and will likely yield a mix of anomeric products (Brameld and Goddard, 1998b).

binding of the -1-boat hexaNAG substrates distorts the sugar residue at subsite a boat conformation (see Figure 1.8), which is not observed for any other GlcNAc residues.

1.7 Structure-Function Studies on the Chitinolytic Enzymes

To date, four crystal structures of family 18 chitinase enzymes have been reported. These include hevamine, a plant defence protein with combined chitinase and lysozyme activity (Anke *et al.*, 1996); chitinase A from *Serratia marcescens* (Perrakis *et al.*, 1994); and endo- β -*N*-acetylglucosaminidases F₁ (endo F₁) from *Flavobacterium meningosepticum* (van Roey *et al.*, 1994) and endo- β -*N*-acetylglucosaminidases H (endo H) from *Streptomyces plicatus* (Rao *et al.*, 1995). In these four structures, the main catalytic domain is an $\alpha\beta$ barrel, which establishes a common fold for all family 18 enzymes (Davies and Henrissat, 1995). They possess a long groove for the binding of the substrate, on the C-terminal edge of the $\alpha\beta$ barrel. The hydrolysis of chitin by chitinases belongs to the general acid-base catalysis proposed for lysozyme.

From primary structure comparisons, mutagenesis, and structural data, it is clear that a glutamic acid and an aspartic acid located at the active site of the catalytic $\alpha\beta$ barrel domain are important to substrate degradation (Vorgias *et al.*, 1996). However, the idea of acid-based catalysis seemed to be contradicted by the most recent mechanism proposed by Brameld and Goddard (1998b) as mentioned earlier in section 1.6.2. They suggested anchimeric stabilisation hydrolysis mechanism (see scheme 3) for family 18 chitinases, and that only Glu315 is involved in the first step of acid-based hydrolysis. A boat conformation of the GlcNAc residue at the binding site where Glu315 will come to react with is also required at the first step. In addition, an oxazoline ion intermediate formed through anchimeric hydrolytic process is assisted by the neighbouring *N*-acetyl group. The second acidic residue (Asp391) appears to play less important role in this mechanism.

With regard to bacterial chitinases, even though the gene level has been investigated extensively, only one publication of the three-dimensional structure of *Serratia*

chitinase (ChiA) is reported (Perrakis *et al.*, 1994). The structure of native ChiA from *Serratia marcescens* has been solved by multiple isomorphous replacement (MIR) and refined at 2.3 Å resolution, resulting in a crystallographic R-factor of 16.2%. In summary, the enzyme comprises three domains: the amino terminal domain (Chi N domain), which consists only of β -strands, connects through a hinge region to the main α/β -barrel domain, and a small domain, which has an $\alpha+\beta$ -fold, is formed by an insertion in the barrel motif (Figure 1.10).

The amino-terminal domain is believed to have an important functional interaction with the filamentous chitin substrate. This postulation is supported by a recent study of Stivil and Kirchman (1998). They investigate the role of ChiN domain by cloning two different versions of the ChiA gene from *V. harveyi*, in which its gene has been isolated and analysed previously (Soto-Gil, 1988). One clone (ChiA1) carries the ChiA gene that expresses both catalytic and chitin-binding domains but another clone (ChiA2) carries the Chi A gene that expresses catalytic domain but lacks the chitin-binding domain. The result clearly shows that the Chi A2 protein does not bind to chitin, but it could hydrolyse chitin, although not as well.

Moreover, apart from being involved in chitin binding, the ChiN domain is also suggested to be important for determination of movement of chitinase along *N*-acetylglucosamine strands and within environments containing chitin. These are proved by slower diffusion of ChiA1 in agarose containing colloidal chitin compared with ChiA2, but no difference in diffusion in agarose without colloidal chitin (Stivil and Kirchman, 1998).

Another study from Perrakis *et al.* (1997) shows that the ChiN domain has structural similarities to fibronectin type III domains (Fn III domains), which exist in other chitinases. However, these two domains have been postulated to have evolved independently in chitinases, as structure comparisons of these two similar domains fail to establish any sequence similarity. Moreover, sequence searches and comparisons between ChiN and FnIII domains show a remarkable difference in their amino acid sequences.



Figure 1.10 Ribbon diagram of ChiA in an orientation to show the three domains and the groove of the active site. The bound sugar is shown in a space-filling model, and the two active site residues are drawn as a ball-and-stick model. β -Strands are represented as arrows, α -helices as spirals. A consensus catalytic region with 13 residues is shown between the asterisk symbols (Perrakis *et al.*, 1994).

The α/β -barrel domain is proposed as the catalytic domain of the enzyme. The substrate-binding cleft is formed by a long groove, located at the C-terminal region of the β -strands of the α/β -barrel. Several pieces of evidence point towards Glu315 that is completely conserved in all the family 18 chitinases, and Asp391 that is highly conserved in the family 18 chitinases, as being involved in enzyme catalysis. These two amino acid residues are also found to be located at the active site of *S. marcescens* ChiA (Figure 1.10).

Comparison of putative catalytic regions in bacteria performed by Svitil and Kirchman (1998) reveals that a consensus catalytic region consists of 13 residues (see Figure 1.10) which are present in at least 69% of 25 bacterial chitinase sequences compared (Figure 1.11). They divide the catalytic domains into at least four groups. Group I (93% mean identity) consists of 4 chitinases, all γ -proteobacteria (Gram-negative). Group II (85% mean identity) includes a chitinase from a γ -proteobacterium and three chitinases from Gram-positive high-G+C-content bacteria. Group III (83% mean identity) is composed of five chitinases all from Gram-negative low-G+C-content bacteria. Group IV (71% mean identity) contains three chitinases from the *Vibrio* family and three chitinases from Gram-positive bacteria. The domains which could not be placed into a group are assigned to Group V (51% mean identity). These comparisons clearly indicate that the evolution of the catalytic region does not follow bacterial phylogeny and that taxonomically related bacteria may have dissimilar catalytic regions, for example three chitinases from a single species (*Aeromonas* sp.) are not very similar (46% mean identity).

The third domain, $\alpha+\beta$ small domain, is formed by an insertion between strand B and helix A near the C-terminal part of the α/β -barrel. The function of this domain still remains unknown.

Based on the known structure of *S. marcescens* ChiA, the three-dimensional structure of *E. agglomerans* ChiA has been modelled and found to have extensive similarities to ChiA of *S. marcescens* (Chernin *et al.*, 1997). Basically, the modelled structure comprises three domains; Fibronectin III like domain, catalytic α/β -barrel domain,

Figure 1.11 Comparison of putative catalytic regions in bacterial chitinases. Conserved residues within a group are in bold. Arrows indicate four highly conserved residues identified by Watanabe *et al.* (1993); *, residue necessary for enzyme activity; @, conserved aromatic residues; γ E, Gram-negative γ -subdivision, family *Enterobacteriaceae*; γ Y, Gram-negative γ -subdivision, family *Vibrionaceae*; β , Gram-negative β -subdivision; lo, Gram-positive low G+C-content; hi, Gram-positive hi G+C-content; Alt A, *Alteromonas sp.* strain O-7 chitinase (accession number D13762; Tsuibo *et al.* 1993b); AeO c, *Aeromonas caviae* chitinase (U09139; Stirit *et al.* 1995); Sma A, *Serratia marcescens* chitinase A (X03657; Jones *et al.* 1986); Egg, *Enterobacter agglomerans* chitinase (U59304; Chernin *et al.* 1997); Sma B, *Serratia marcescens* ChiB (X15208; Harpster & Dunsmuir. 1989); Stm 63, *Streptomyces plicatus* chitinase 63 (M82804; Robbins *et al.* 1992); Stm C, *Streptomyces lividans* chitinase C (D12647; Fujii & Miyashita 1993); Stm 40, *Streptomyces thermoviolaceus* chitinase 40 (D14536; Tsujibo *et al.* 1993a); Bcc A1, *Bacillus circulans* WL-12 chitinase A1 (J05599; Watanabe *et al.* 1990); Bcl, *Bacillus licheniformis* TP chitinase (U71214; Tantimavanich & Panbangred, 1996); Bct, *Bacillus thuringiensis* chitinase (U89796; Wiwat *et al.*, 1996); Clt A, *Clostridium thermocellum* ChiA (Z68924); Kuz, *Kurthia zopfii* chitinase (D63702; Toyoda, 1995); ChiA, *Vibrio harveyi* BB7 chitinase A (Svitil & Kirchman 1998); AeO 1 and 3, *Aeromonas sp.* NO. 10S-24 chitinase ORF 1 and 3, respectively (D63139, D63141; Shiro *et al.* 1996); Bcc D, *B. circulans* WL-12 chitinase D (D90534; Watanabe *et al.* 1992); Stm X, *Streptomyces olivaceoviridis* exochitinase (X71080; Blaak *et al.*, 1993); StmA, *St. lividans* 66 ChiA (D13775; Miyashita & Fujii 1993); AeO II, *Aeromonas sp.* NO. 10S-24 chitinase II (D31818; Ueda *et al.* 1994); AeO 2, *Aeromonas sp.* NO. 10S-24 chitinases ORF 2 (D63140; Shiro *et al.* 1996); Ewa, *Ewingella americana* chitinase (X90562); Jan, *Janthinobacterium lividum* chitinase chitin-binding domain 2 (U07025; Gleave *et al.* 1995); Bcc C, *B. circulans* WL-12 ChiC (D89568; Alam *et al.* 1995); Sae, *Saccharopolyspora (Streptomyces) erytraeus* chitinase (P14529; Kamei 1989).

					↓	↓			↓	*	
I	γV	Alt A	261	PDLKILPSVGGWTLSD-----	PFHGFTNKAN-RDTFVASVKQFLKT--WK-F--	YDGV	DIDWE	FPGGD			
(93%)	γV	Aeo c	263	PDLKILPSVGGWTLSD-----	PFYFLGDKTK-RDTFVASVKEFLQT--WK-F--	FDGV	DIDWE	FPGGQ			
	γE	Sma A	263	PDLKILPSIGGWTLSD-----	PFFFMGDKVK-RDRFVGSVKEFLQT--WK-F--	FDGV	DIDWE	FPGGK			
	γE	Egg	263	PDLKILPSIGGWTLSD-----	PFFFMGDKVK-RDRFVGSVKEFLQT--WK-F--	FDGV	DIDWE	FPGGG			
II	γE	Sma B	85	PSLRIMFSIGGWYYSNDLGVSHANYVNAVKT	PAARTKFAQSCVRIMKD--YG----	FDGV	DIDWE	YPQAA			
(85%)	hi	Stm 63	330	PHIKILYSFGGWTSWGGF-----	PDAVKNPAAFAKSCHDLVEDPRWADV--	FDGID	LDWE	YPNAC			
	hi	Stm C	329	PNIKILYSFGGWTSWGGF-----	PDAVKNPAAFAKSCHDLVEDPRWADV--	FDGID	LDWE	YPNAC			
	hi	Stm 40	137	PHIKVLWSFGGWTSWGGF-----	ADAAKRPAAFQAQSCYNLVHDP	PRWDGV--	FDGID	--WEYPNAC			
III	lo	Bcc A1	52	PNLKTIIISVGGWTWSN-----	RFSDVAATAATREVFANS	AVDFLRK--YN----	FDGV	DLDE	WYFPVSG		
(83%)	lo	Bcl	149	PHLKTFIISVGGWTWSN-----	RFSDVAADPVARGNFAASAVEFLRK--YG----	FDGV	DLDE	WYFPVSG			
	lo	Bct	157	PHLKTIIISVGGWTWSN-----	RFSDMAADEKTRKVF	AESTVAFLPA--YG----	FDGV	DLDE	WYFPGVE		
	lo	Clf C	112	PHVKTILISVGGWTESK-----	YFSDVALTEESRNTFADSCVEFIRT--YR----	FDGV	DIDWE	YFPVSG			
	lo	Kuz	153	PNLKTIIISVGGWTWSN-----	RFSDVAATAATREVFANS	AVDFLRK--YN----	FDGV	DLDE	WYFPVSG		
IV	γV	ChiA	110	QGRSVLLALGG-----	ADAHVELETGDE-R-AFADE---	IRLTER	YG----	FDGL	DIDLE	QAAVT	
(71%)	γV	Aeo 1	302	RGKKVLISIGG-----	QNGEIQLTTTAA-RDNFVN	SISAIIDK--WS----	LDGL	DIDF	EGHSLQ		
	γV	Aeo 3	318	KGKKVLSLGG-----	QDGSITLNTASD-GDNFVN	SLYGILTK--YG----	FDGID	LDLE	RAARE		
	lo	Bcc D	254	QGKKVLISMGG-----	ANGRIELTDATKKRQ	QFEDSLKSIIST--YG----	FNGL	DIDLE	GSSLS		
	hi	Stm X	336	AGKKVLISIGG-----	QNGQVQLTTTAA-RDTFV	SSVSKIIDE--YG----	LDGL	DIDF	EGHSLS		
	hi	Stm A	350	AGKKVIISVGG-----	EKGTVSVNSSAS-ATNFAN	SVYSVMRE--YG----	FDGV	DIDLE	ENGLNP		
V	γV	Aeo II	96	QGGRLIISFGG-----	AAVPMWKPAPVAPRWRP	WMPCCNAPACV-----	PLD	FDIE	GSQLS		
(51%)	γV	Aeo 2	285	LGGGVIISSGGWNASD-----	IVRTCTDAR-----	SIATVYENVLER-FGA-	DHLD	SDPE	HGDQE		
	γE	Ewa	80	ANRQVIVSFGG-----	ASNADISTKFTVDQLVQTYTDV	VQKFKAKQ-----	LD	FDLE	NGQYD		
	β	Jan	357	PNLKLFIISLGGWSWSKNF	SVGSATDALRKQMVRS	CIDIYIKGNLPVQGGVGAAANI	FDGID	DIDWE	YPVGG		
	lo	Bcc C	104	PDLKVLLSVGGWG-----	ANGFSDAALTDASRTTFAD	SIVQLVTSNN-----	LDGV	DLDE	WYPTNP		
	hi	Sae	69	AGGDVIPSIGGYSGSKL-----	GEVCQDSQSLAGAYQK-	VIDAYGLK-----A---	IDV	DIE	ATEFE		
consensus				K	S	GG		F	S	@	FDG D D E

and α + β small domain. Glu315 and Asp391 are also found to be located at the active site of the α / β -barrel. The functions of each domain are therefore proposed to be the same.

1.8 Applications

Chitinase is a key enzyme for the degradation of chitin, which is found to be abundant throughout nature and serves as a major component of fungi and as exoskeletons of insects and crustaceans such as shrimps, and crabs. Therefore, chitinases have been studied from various points of view, but they show three main applications as follows:

1.8.1 Agriculture

Several indications as mentioned previously in section 1.5 suggest that chitinases play a defence role against plant pathogens. This seems to be of great advantage for the improvement of crop plants with high commercial value i.e. potato, rice, barley, beans, etc. Several strategies can be employed. For example, development of transgenic plants with chitinase activity against fungal pathogens or chitinase-sensitive parasites, or DNA manipulation for obtaining higher expression levels of the enzyme for anti-pathogen application.

Ethylene has been suggested as an effective inducer for production of enzymes, especially chitinases, involved in pathogen resistance in plants. A report (Boller *et al.*, 1983) showed that ethylene induced endochitinase production in bean leaves *Phaseolus vulgaris* (L.), and effects of the ethylene-induced enzyme on fungal growth were examined. It was found that apart from detection of liberation of chitin in isolated cell walls of a plant fungal pathogen *Fusarium solani*, the enzyme also acted as a lysozyme when incubated with *Micrococcus lysodeikticus*.

A study by Lorito *et al.* (1993) indicates from another point of view that the chitinolytic enzymes from *Trichoderma sp.* are inhibitory to a wider range of

deleterious fungi than similar enzymes from other sources. In addition, combinations of enzymes are much more effective than single enzymes; while 50% lethal dose (ED₅₀) values for single enzymes range from 40 to 120 µg/ml, the ED₅₀ values for three chitinolytic enzymes combined were only about 3 µg/ml of total protein. Further, these enzymes are synergistic with various synthetic fungicides and with the biological bacterium *Enterobacter cloacae*. Consequently, this synergistic complex of enzymes is of substantial interest for the production of transgenic plants with resistance to plant pathogens and microbes.

Disease resistance in transgenic plants has been improved for the first time by insertion of a gene from a biocontrol fungus (Lorito *et al.*, 1998). The gene encoding a strongly antifungal endochitinase was transferred from the mycoparasitic fungus *Trichoderma harzianum* to tobacco and potato, and substantial differences in endochitinase activity were detected among transformants. Selected transgenic lines were highly tolerant or completely resistant to the foliar pathogens: *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and the soil-borne pathogen *Rhizoctonia solani*. The high level and the broad spectrum of resistance obtained with a single chitinase gene from *Trichoderma* overcame the limited efficacy of transgenic expression in plants of chitinase genes from plants and bacteria. This study demonstrates a rich source of genes from biocontrol fungi that can be used to control diseases in plants.

In chitinolytic bacteria, chitinases show application in terms of biocontrol agents of fungal pathogens. For example, Cronin *et al.* (1997) reported that all the chitinase-producing bacteria isolated from soil-borne reduced the egg hatch of the potato cyst nematode *Globodera rostochiensis* with strong effects in some isolates (up to 90% inhibition compared with the controls). The antifungal activity of *E. coli* JM109 carrying the *Enterobacter agglomerans* endochitinase was demonstrated in vitro from a recent study (Chernin *et al.*, 1997). They indicated that the cloned endochitinase inhibited spore germination of *Fusarium oxysporum*. Moreover, the transformed strain inhibited both *Rhizoctonia solani* growth on plates and the root disease caused by this fungus in cotton seedlings under greenhouse conditions. In addition, a chitinolytic isolate of *Aeromonas caviae* and partially purified chitinase produced by

the cloned gene from *Serratia marcescens* have also proved to be effective biocontrol agents (Inbar and Chet, 1991; Shapiro *et al.*, 1989).

1.8.2 Biomedicine

Since the natural chitinase substrate does not frequently occur in vertebrates, this has led some authors to propose chitin metabolism as a potential drug target for the treatment of some forms of human parasitosis (Splindler *et al.*, 1990; López-Romero and Villagómez-Castro, 1993; Shahabuddin and Kaslow, 1993; and Fuhrman, 1995) and fungal infections (Georgopapadakou and Tkacz, 1995). An initial study was conducted with crude amoeba homogenates (Julio *et al.*, 1996). It was shown that at least three forms of chitinase (ChiA, ChiB, and ChiB') existed in *Entamoeba invadens* and these amoeba chitinases responded to allosamidin (Figure 1.12), that is an analogue of diacetylchitobiose consisting a dimer of *N*-acetylallosamine joined to an aminocyclitol (Sakuda *et al.*, 1986). It serves as a strong specific inhibitor of chitinase from diverse sources, with different kinetics. According to the K_m/K_i ratios, the A and B' forms showed 450- and 350-fold higher affinity for the inhibitor over 4-MU-(GlcNAc)₃ substrate, whereas for the B form is as high as 5130-fold. It was speculated that the enzymes may be required during encystation for the modelling of chitin chains during cyst wall elaboration and may be involved in wall softening to allow release of the metacyst during the poorly understood process of excystation. From biochemical properties with emphasis on the kinetics of inhibition by allosamidin, it was assumed that results obtained with *E. invadens* could later be extrapolated to the human parasite *E. histolytica* due to their similarities in morphology and life cycles.

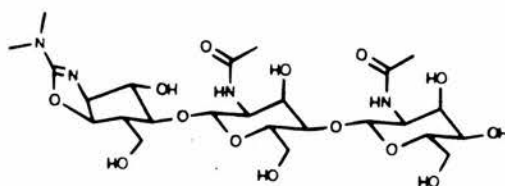


Figure 1.12 The structure of allosamidin, an diacetylchitobiose analogue

In addition, prior studies indicated that a microfilarial stage-specific chitinase is a possible candidate antigen for a transmission-blocking vaccine against filarial nematodes such as *Brugia malayi* and *Wuchereria bancrofti*, which infect nearly 80 million people world-wide and are major causes of morbidity and economic loss in endemic areas. Effective drugs or for novel control strategies such as vaccines has been paid more attention. One possible approach would be to interrupt transmission by reducing the number of microfilariae in the blood of the human population and/or interfering with the parasite molecules that appear to be necessary for the penetration of the mosquito midgut and the subsequent development of microfilariae in the insect.

Evaluation of recombinant chitinase for use in anti-microfilarial vaccines was reported recently by Wang *et al.* (1997). Their results indicate that immunisation of jirds with recombinant fusion proteins containing all or only the C-terminus of parasite chitinase induced partial protection against microfilaremia resulting from subsequent infection with *Brugia malayi*. The protective epitope appears to be located close to the C-terminus of the chitinase molecule.

1.8.3 Biological Conversion Process

Since chitin is one of the most abundant organic substance in nature and many billion tons of this polysaccharide are deposited annually on the ocean floor, many attempts to achieve the conversion of significant amounts of this insoluble molecule into usable forms via biological processes have been increasingly considered. Development to obtain higher levels of chitinase expression for exploitation of chitin for commercial uses has been therefore attempted. Extensive studies have been focused on chitinases from various microorganisms (Monreal and Reese, 1968; Tsujibo *et al.*, 1992; Takahashi *et al.*, 1993; Ueda *et al.*, 1994; Huang *et al.*, 1996; Hiraka *et al.*, 1997; and Svitil *et al.*, 1997). The most complete study is that of ChiA from *Serratia marcescens*, in which investigation of its gene is well established and the three-dimensional structure of the enzyme is already solved. Moreover, the mechanism of the enzyme on chitin degradation has also been proposed recently.

However, chitinases from marine bacteria especially from the family *Vibrionaceae* have been the targets of extensive interest during the current decade. Nevertheless, all of the work is still limited to the laboratory level, and has been concentrated on the studies of the enzyme molecule itself in order to understand the relationship of structure and enzyme functions. For example, a report was published by a group of researchers in Japan (Osawa and Koga, 1995). They found that 48 aquatic bacteria isolated from the genus *Vibrio* apparently utilise chitin as the sole source of carbon and nitrogen for growth. However, the bacterial growth is much faster with GlcNAc than with chitin, indicating that the enzymatic breakdown of chitin (by chitinase and chitobiase) to GlcNAc limits the growth rate. It is suggested that in aquatic environments that are often nutritionally competitive, the likelihood of an individual bacterium encountering readily metabolisable substrates would extremely small. In this situation, their ability to degrade chitin to GlcNAc is certainly an important nutritional strategy, but the ability to utilise GlcNAc as a sole source of nitrogen is equally important.

In 1997, Svitil and colleagues (Svitil *et al.* 1997) examined chitin-degrading proteins in the marine bacterium *Vibrio harveyi* and found that the bacterium has a higher growth rate and more chitinase activity when grown on β -chitin (isolated from squid pen) than on α -chitin (isolated from snow crab). This is suggested to be a result of more open structure of β -chitin that allowed the enzyme better access. In addition, when grown on different types of chitin, *V. harveyi* excretes several chitin-degrading proteins (more than 10 chitinases) into the culture medium. This is suggested to be for the bacterium to utilise different forms of chitin more efficiently as their nutritional source. This clearly implies that multiple forms of chitinase need to be studied and produced for a complete treatment of chitin waste in the actual field.

The report of using chitinase for treatment of chitin waste was published by Carroad and Tom (1978). They reported utilising the extracellular chitinase system of *Serratia marcescens* to hydrolyse the chitin fraction of the waste. Because of similarities in their chemical structures, the process design for bioconversion of

chitin was largely based on the process development for bioconversion of cellulose to ethanol and single-cell protein.

The process conceptualises the bioconversion of chitin wastes into single-cell protein suitable for animal feed or aquaculture feed. In principle, a four-step process was envisioned as a maximum flexibility in chitin utilisation (Figure 1.13). In the first step the chitin waste receives some pre-treatments which involve drying and size reduction. Chemical purification measures may also be required as the chitin waste contains considerable amounts of protein and calcium carbonate. In the second step, a small fraction of the waste is diverted to an enzyme production compartment. Chitin waste is used to induce a selected microorganism to secrete an extracellular chitinase system into solution in submerged culture. In the third step, the enzyme is harvested by filtration and combined with the bulk of the chitin waste for chitin hydrolysis. The chitin is enzymatically hydrolysed, mainly to the monomer (GlcNAc) and its dimers (GlcNAc₂). The hydrolysate is filtered free of undigested solids and, in the fourth step, is fed to a product generation stage where the hydrolysis products are fermented in submerged culture to single-cell protein by an appropriate microorganism. Product purification may follow as necessary.

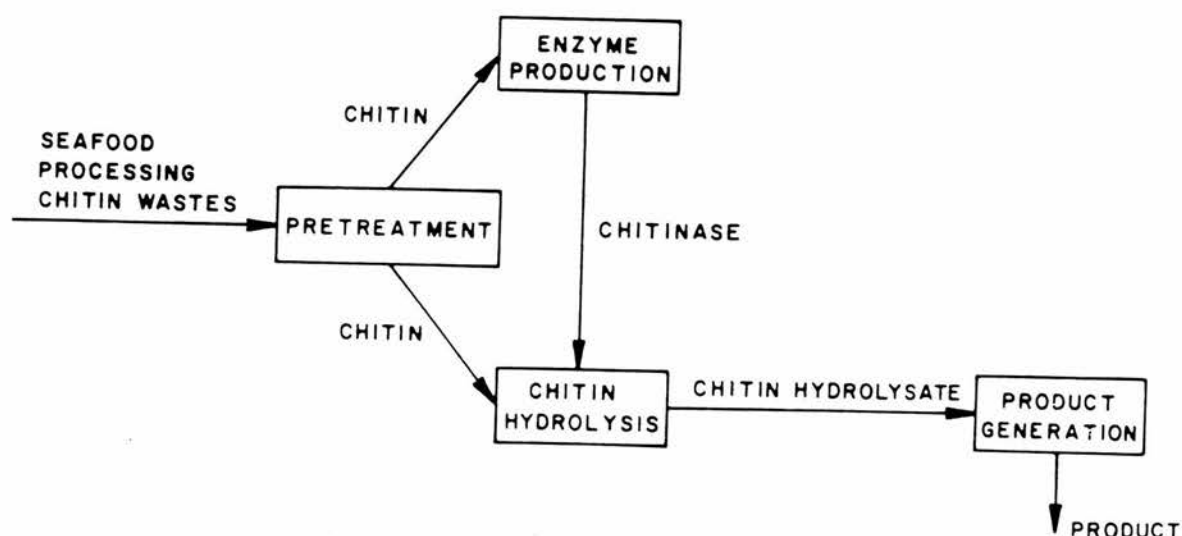


Figure 1.13 Four-step process concept for chitin bioconversion.

Alternatively, if the enzyme production, hydrolysis, and production steps are combined into a single vessel, the process could be reduced to two steps as shown in Figure 1.14. The two-step process would be applied if the organism producing chitinase had value as a protein source, or if the desired product was the chitinase system, or if the product-generating organism could be co-cultured in the same fermentor.

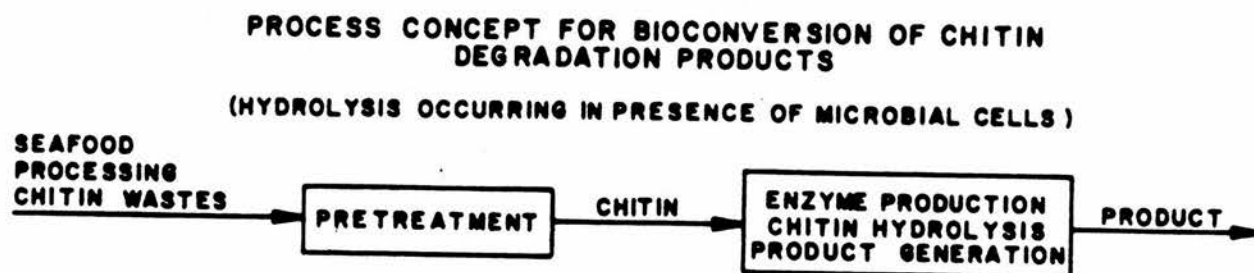


Figure 1.14 Two-step process concept for chitin bioconversion.

It was suggested from Carroad's study (Cosio *et al.*, 1982) that enzymic hydrolysis of pretreated chitin waste achieved 80% conversion in 24 h. Optimum conditions were also determined for maximum chitinase production in submerged culture, using pretreated chitin as substrate.

Carroad and his research team (Cosio *et al.*, 1982) also published an economic analysis of a process for the bioconversion of shellfish chitin waste, which included information on waste pre-treatment and chitinase production. The integrated process scheme for conversion of shrimp shell chitin waste to yeast single-cell protein based on their studies was designed and analysed economically, giving a negative after-tax cash flow (using a 48% tax rate and straight-line depreciation over a seven year asset life) of \$0.06 per kg wet waste. A summary of the economic analysis of shrimp shell bioconversion process is shown in Table 1.4

Table 1.4 Summary of economic analysis of shrimp shell bioconversion process (Cosio *et al.*, 1982).

Distribution of costs Among process inputs		Distribution of costs among major steps	
Depreciation	41.9%	Size reduction	6.5%
Materials	15.0%	Deproteination and recovery	36.4%
Labour	14.2%	Demineralisation	11.1%
Utilities	2.3%	Chitinase production	12.0%
Maintenance	8.8%	Chitin hydrolysis	8.7%
Other	17.8%	Single-cell protein production	10.8%
		Other	14.5%
Total	100%	Total	100%

Total cost = \$10,900 per 6 month season and 1,092 tons of chitin waste.

Depreciation is calculated over 7 yr; no investment tax credit assumed

1.9 Literature Search for Chitinase Studies

Bacterial chitinases have been extensively studied from various points of view during the past few years. The result obtained from Bath Information and Data Services (BIDS) search clearly shows that the study of bacterial chitinases has been dramatically increasing each year. To date, there are 79 hits, in which 50 articles were published during the past three years. Recent chitinase studies are mainly directed towards investigation of genes encoding chitinase for determination of structure-function relationship, and development of expression system for exploitation of chitin in nature. A summary of bacterial chitinase studies is given in Appendix VII.

1.10 Aim of Project

My project is concerned with the study of chitinase enzymes from the marine bacteria *Vibrio* at the molecular level. The reason for interest in this enzyme is to exploit it for recycling chitin waste into a commercially usable form. This type of waste is released in huge amounts by the manufacturing processes of the shrimp and crab industry in Thailand. Marine bacteria *Vibrio* are suggested to have a high potential in this application. However, there are only a few studies dealing with the enzyme at the molecular level. The relationship between polysaccharide degrading function and the molecular structure is still unclear. My work done over the past three years has accomplished five aims as follows:

1. Screening for marine bacterium *Vibrio* producing high level of chitinase activity.
2. Purification and partial *N*-terminal amino acid sequencing of the purified chitinase from *V. carchariae*
3. Isolation, sequencing, and expression of the gene encoding chitinase from *V. carchariae*
4. Localisation and protein processing studies of chitinase in *E. coli* system
5. Molecular structural study of *V. carchariae* chitinase based on the known structure of *S. marcescens* chitinase A

However, further work need to be carried out in order to achieve the long term goal of the complete project as follows:

6. Development of a high expression and secretion system in *E. coli* to provide sufficient enzyme to detailed characterisation and for crystallisation by genetic engineering.
7. Site-directed mutagenesis studies to extend our understanding of the mode of action of this enzyme.
8. Introduction of the chitinase gene into a suitable bacterial expression system for the recycling of chitin which is a by-product of the shrimp industry in Thailand.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Determination of Chitinase Activity

Chitosan, chitin flakes from crab shells, glycol-chitosan, chitin-azure, 4-methylumberriferryl-*N,N'*- β -D-diacetylchitobioside, 4-methylumberriferryl-*N,N',N'',N'''*-tetraacetyl- β -D-chitotetraoside, chitinase from *Serratia marcesens* were supplied by Sigma Chemical Company. [^{14}C]acetic anhydride, solubilised in toluene solution, 19mCi/mmol was kindly provided by Professor Stephen C Fry, Institute of Cell and Molecular Biology, University of Edinburgh. [^3H]acetic anhydride, stabilised in toluene solution, 0.5 mCi/mmol was purchased from Amersham plc, Lincoln Place, Aylesbury, Buckinghamshire.

2.1.2 Bacterial Strains

Fourteen type strains of marine *Vibrio* for chitinase activity were obtained from three culture collections (LMG, Laboratorium voor Microbiologie Gent, Rijksuniversiteit, Gent, Belgium; NCIMB, National Collections of Industrial and Marine Bacteria, Aberdeen, Scotland, U.K.; ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.): *V. aestuarianus* (LMG7909^T); *V. alginolyticus* 283 (LMG4408^T = ATCC17749 = NCIMB1903); *V. alginolyticus* 284 (LMG4409^T), *V. campbellii* (LMG11216^T), *V. carchariae* (LMG7890^T), *V. diazotrophicus* (LMG7893^T), *V. fischeri* (LMG4414^T = NCIMB1281 = ATCC7744); *V. gazogenes* (LMG13541); *V. harveyi* (LMG4044^T = NCIMB1280 = ATCC14126); *V. marinus* (NCIMB1144); *V. natriegens* (LMG10935^T); *V. nereis* (LMG3895^T = NCIMB1897 = ATCC25917); *V. pelagius* (LMG3897^T = NCIMB1900 = ATCC259160); *V. splendidus* (LMG4042^T =

NCIMB1 = ATCC33125). The bacterial strains were supplied from the laboratory of Professor Brian Austin, Department of Biological Sciences, Heriot-Watt University.

E. coli type strain XL1 blue kindly provided by Dr. Peter Estibeiro, Department of Biochemistry, University of Edinburgh, was used for gene cloning.

2.1.3 Bacterial Growth

Bacteriological peptone, yeast extract, and agar bacteriological (Agar No 1) were purchased from Oxoid Unipath Ltd., Basingstoke, Hampshire, England. Sea water was from the North Berwick seashore.

2.1.4 Protein Purification

Ammonium sulphate was supplied from Sigma Chemical Company. DEAE-Sephadex A-50 resin was a product of Whatman International Ltd. Sephacryl-S200-HR resin was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Superose 12[®] HR 10/30 column for fplc, and MonoQ column for fplc were from Pharmacia LKB Biotechnology AB, Uppsala, Sweden.

2.1.5 Carbohydrate Analysis

Glucosamine, *N*-acetylglucosamine, silver nitrate, lysine, and *p*-hydroxybenzoic acid hydrazide were products of Sigma Chemical Company. Methylene blue, chlorobutanol, acetic anhydride/pyridine solution, and thiosulfate solution were kindly provided by the laboratory of Professor Stephen C Fry, Institute of Cell and Molecular Biology, University of Edinburgh.

2.1.6 Antibody Production

Polyclonal antibodies raised against *V. carchariae* chitinase were produced by the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, Scotland. Titermax[®] Gold adjuvant was a kind gift of Dr. Richard Ashley, Department of Biochemistry, University of Edinburgh.

2.1.7 Western Blotting

Hyperbond[™] -C pure nitrocellulose membrane and ECL Western blotting detection reagents were supplied by Amersham Life Science International plc, Lincoln Place, Aylesbury, Buckinghamshire. Anti-Rabbit IgG (whole molecule) Peroxidase Conjugate, and BioMax MS-1 film were purchased from Sigma Chemical Company.

2.1.8 Plasmids

pGEM-T plasmid was supplied by Promega Cooperation, Madison, U.S.A.. pBluescript II KS(-) plasmid was a kind gift of Dr. Peter Estibeiro, Department of Biochemistry, University of Edinburgh.

2.1.9 Oligonucleotides

Three degenerate oligonucleotides designed from the *N*-terminal amino acid sequence of *V. carchariae* chitinase and universal oligonucleotides; M13, M13(rev), and SP6 for PCR work and manual sequencing were ordered from MWG-Biotech GmbH Company. All the oligonucleotides for automatic DNA sequencing were designed and supplied by Oswel Research Products Ltd., Southampton, England.

2.1.10 DNA Isolation and Purification

DNA extraction kit, QIAEX II, Plasmid DNA purification kit, and QIAGEN Maxi kit, were supplied from QIAGEN, Germany. Phenol/Chloroform/Isoamyl alcohol solution, spermadine, and proteinase K were supplied by Sigma Chemical Company. GELase was a product of Epicentre Technologies.

2.1.11 Enzymes for DNA Manipulation

All the restriction enzymes used for DNA digestion were supplied from New England Biolab Ltd., except *EcoR* I and buffers were products of Boehringer Mannheim GmbH, Germany. Taq polymerase was a product of Biolab Ltd., Jerusalem, Israel. T₄ DNA ligase, and alkaline phosphatase were purchased from Promega Cooperation, Madison, U.S.A.

2.1.12 DNA Sequencing

SequenaseTM PCR Product Sequencing kit, Sequenase[®], deoxyadenosine 5'-[α -³⁵S] thiotriphosphate, stabilised in buffered solution, 1,000Ci/mol, and Hyper film MP X-ray film were supplied by Amersham plc, Lincoln Place, Aylesbury, Buckinghamshire. Ready Sol DNA/PAGE, 40% (w/v) stock solution, and Repel-Silane ES solution were purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Automated double-stranded DNA sequencing of the entire chitinase gene from *V. carchariae* was performed by Oswel Research Products Ltd., Southampton, England.

2.1.13 Miscellaneous

General reagents: acetic acid, acetone, and glycerol were purchased from Sigma-Aldrich Co. Ltd., Gillingham, Dorset, U.K. Chloroform, DMSO (dimethylsulfoxide), hydrochloric acid, ortho phosphoric acid, TCA (trichloroacetic acid), TFA

(trifluoroacetic acid), toluene, and Triton X-100 were supplied from BDH Chemicals Ltd., Poole, England. Methanol and absolute ethanol were supplied from Fisher Scientific UK Ltd., Bishop Meadow Road, Loughborough. DMF (*N,N'*-dimethylformamide), isopropyl alcohol, and isoamyl alcohol were purchased from Sigma Chemical Company. Ultima GoldTM high flash point LSC-cocktail for aqueous and non-aqueous samples (a product of Packard, A Canberra Company) was kindly provided by the teaching laboratory, Department of Biochemistry, University of Edinburgh.

General chemicals: D-(+)-glucose, sucrose, sodium chloride, ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside), lysozyme, PMSF (phenylmethyl sulfonylfluoride), BSA (bovine serum albumin), ovalbumin (chicken fraction V), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), phosphorylase b, guanidine hydrochloride, POPOP (1,4-bis[5-phenyl-2-oxazolyl]benzene-2,2'-p-phenylene-bis[5-phenyloxazole]), PPO (2,5-diphenyloxazole), EDTA (ethylenediamine tetraacetic acid), 2-mercaptoethanol, ammonium persulphate, High molecular weight standard protein mixture, potassium phosphate (monobasic), potassium phosphate (dibasic), sodium carbonate, sodium acetate, MES (2-[*N*-morpholino]ethanesulfonic acid), Trizma®base (tris[hydroxymethyl]aminomethane), sodium dodecyl sulphate (SDS), TEMED(*N,N,N',N'*-tetramethylethylenediamine), agarose type XI (low gelling temperature) were supplied from Sigma Chemical Company.

Glycine, sodium hydroxide, IPTG (isopropyl- β -D-thiogalactopyranoside), Coomassie brilliant blue G-250, and bromophenol blue were supplied by BDH Chemicals Ltd., Poole, England.

Urea, agarose (ultrapure grade), and 1 kb DNA ladder were purchased from Promega Cooperation, Madison, U.S.A.

30% (w/v) Acrylamide/bis-acrylamide stock solution (37.5:1 by vol.) was a product of Anachem Ltd., Luton, Beds.

Ethidium bromide was a product of International Biotechnologies, Inc., New Haven, Connecticut, U.S.A.

Low molecular weight standard protein mixture was a product of Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Dried skimmed milk was a product of Sainsbury's Supermarket Ltd., Stamford Street, London.

2.2 Methods

Except for those given in this section, the recipes for bacterial growth media, and all the common reagents and buffers that were used in this study are described in appendices.

2.2.1 Development of Assay Method for Chitinase Studies

2.2.1.1 Determination of Chitinase Activity by Radioactive Assay

2.2.1.1.1 Preparation of Radiolabelled Chitin Substrate

Preparation of [^{14}C]chitin and [^3H]-chitin was performed according to the procedure for [acetyl ^3H] chitin described by Cabib (1988). Tritiated chitin was obtained by acetylation of chitosan with [^3H]acetic anhydride. Chitosan (1g) was ground in a mortar while adding slowly a small portion (20 ml) of 10% (v/v) acetic acid, until a syrupy solution was obtained. The chitosan solution was covered with a sheet of parafilm and allowed to stand overnight at room temperature to complete dissolution of the polysaccharide. The next day, 90 ml of methanol was added slowly with mixing and the cloudy solution was filtered through glass wool on a Büchner funnel. The filtrate was placed in a beaker on a magnetic stirrer and 1.5 ml of acetic anhydride, containing 100 μl [^3H]acetic anhydride, 20 mCi (specific activity 500 mCi/mol), was

added and stirred until the magnet came to a stand still. The gel was allowed to stand for about 30 min and then cut up into small pieces with a spatula. The liquid that oozes out was removed and the gel fragments were transferred to the cup of a motor-driven homogeniser. After covering with methanol, the suspension was homogenised for 1 min at maximum speed. The finely divided chitin was centrifuged at 10,000 rpm at 4°C for 15 min and the supernatant was removed. The chitin was washed extensively with water until the filtrate was free of radioactivity. The chitin was resuspended in water to obtain a final concentration of 15 mg/ml, and used as substrate in the chitinase assay.

2.2.1.1.2 Measurement of Chitinase Activity

[¹⁴C]Chitin was initially used to evaluate the radioactive assay. However, in all other subsequent studies, a [³H]chitin substrate was used. In a total volume of 100 µl, 15 µl of [¹⁴C]chitin or [³H]chitin was added with continuous stirring to the reaction mixture containing 5 µl of 1 M MES buffer, pH 6.0, and 80 µl of diluted *S. marcescens* chitinase solution (6 mU). The reaction was incubated at 30°C for 1 h in a shaking waterbath before the reaction was terminated by addition of 300 µl 10% (w/v) TCA. The assay solution of 200 µl was transferred to a microfuge tube, and centrifuged at maximum speed (13,000 rpm) of the bench top centrifuge for 5 min at room temperature. Then 100 µl of supernatant was transferred to a scintillation insert vial containing 150 µl distilled water and 2.5 ml of scintillation cocktail. The radioactivity was measured using a Packard Tri-CARB Liquid Scintillation Analyzer model 1900 CA.

2.2.1.2 Determination of Chitinase Activity by Viscometric Assay

In this study, glycol-chitin, prepared from glycol-chitosan, was used to determine the chitinase activity. Decrease of viscosity of glycol-chitin with times by the action of *S. marcescens* chitinase was measured in a viscometer as mentioned in section 2.2.1.2.3.

2.2.1.2.1 Preparation of Glycol-chitin

Glycol-chitin was prepared from glycol-chitosan by modifying the method for preparation of radiolabelling chitin. Glycol-chitosan (420 mg) was dissolved in 8.4 ml acetic acid, then 37.8 ml methanol was added. At this step, the glycol-chitosan became viscous. Acetic anhydride (0.63 ml) was added into the stirred solution and further stirred until the solution became gelatinous, then resuspended with two volumes of hot distilled water. The acetylated chitin suspension was dialysed against 3 x 2000 ml distilled water for 2h at 4°C, then 2,000 ml distilled water containing 0.5% chlorobutanol overnight at 4°C, and used for determination of chitinase activity by the viscometric method.

2.2.1.2.2 Determination of Degree of Acetylation of Glycol-chitosan

Glycol-chitin was prepared in the same manner as mentioned in section 2.2.1.2.1 but 20 mg of glycol-chitosan was used as the initial material. After preparation, a final volume of 5.0 ml of dialysed glycol-chitin was titrated with 10 mM NaOH to bring the pH from 5.8 to 8.9. The final volume of NaOH after titration was observed in order to determine the percentage of acetylation.

2.2.1.2.3 Measurement of Chitinase Activity

The reaction mixture (0.5 ml) contained 0.25 ml of 4% (w/v) glycol-chitin solution, variable amounts of *S. marcescens* chitinase; 2 mU, 4 mU, 8 mU, and 16 mU, and 1M MES buffer, pH 6.0. The reaction was incubated at room temperature in a viscometer No 42, and the flow time of the reaction mixture was measured at time intervals from 1 min to 360 min.

2.2.1.3 Determination of Chitinase Activity by Colorimetric Assay

Colorimetric assay for chitinase activity was determined towards chitin-azure substrate according to the method suggested by Hackman and Goldberg (1964). Chitin-azure powder was resuspended in distilled water to obtain a concentration of 1%. The reaction mixture comprised 250 μ l of chitin-azure suspension, variable units of *S. marcescens* chitinase (2 mU, 4 mU, 8 mU, and 16 mU) and 5 μ l of 1M Mes buffer, pH 6.0, and distilled water to a final volume of 0.5 ml. Each reaction mixture was incubated at time intervals of 0 min, 10 min, 20 min, 30 min, 60 min, 120 min, 240 min, and 480 min at 30°C before the reaction was stopped by addition of 0.5 ml of 10 % (v/v) TCA. The reaction was performed in duplicate. Absorbance at 520 nm was determined. The λ_{max} of chitin-azure was determined by addition of 1 ml concentrated sulphuric acid into 500 μ l of 1% (w/v) chitin-azure to cleave the bond formed between chitin and azure molecules. The unsolubilised substrate was removed by centrifugation at 5,000 rpm for 5 min at room temperature. The supernatant containing azure products was measured spectrophotometrically at a visible wavelength from 330 to 600 nm.

2.2.1.4 Definition of Chitinase Activity

Measurement of the specific radioactivity of [^3H]chitin after preparation was determined in triplicate by taking 50 μ l of [^3H]chitin suspension into microfuge tubes and drying in vacuo. The dried [^3H]chitin was weighed, and the mole content of chitin was calculated from equivalent mole of monomer unit, *N*-acetylglucosamine (GlcNAc).

1 Unit of chitinase (U) is defined as the amount of enzyme that releases 1 μ mol of [^3H]GlcNAc. Unit of *S. marcescens* chitinase supplied by Sigma was obtained from the information enclosed with the product.

2.2.2 Expression of Chitinases in Marine Bacteria *Vibrio*

2.2.2.1 Bacterial Growth and Storage

For the screening experiments, all the *Vibrios* were grown in Vibrio Complex Medium (VCM), pH 8.5, except that *V. carchariae*, *V. marinus*, and *V. splendidus* were grown in marine medium, pH 7.6. All the species were incubated at 30°C except *V. marinus* and *V. splendidus* that were grown at 20°C. For further work, the bacteria were grown in marine medium, pH 7.6. For small-scale preparation, single colonies of the marine *Vibrio* were picked and grown overnight in universal bottles containing 5 ml of an appropriate medium with 200 rpm shaking at appropriate temperature. For large-scale preparations, the freshly grown cultures were transferred to 250-ml or 500-ml or 1000-ml flasks containing desired volume of the culture medium. Conditions for growing cells were as same as described for small-scale preparation. For short-term storage, the bacteria (freshly subcultured) were streaked on marine agar plates containing 5% (w/v) swollen chitin. After the plates were incubated overnight at 30°C, they were stored at 4°C, and were subcultured and re-streaked every two months. For long term storage, 500-μl aliquots of the cultures were added with an equal volume of 30% (v/v) glycerol, and kept at -70°C.

2.2.2.2 Preparation of Swollen Chitin

Swollen chitin was prepared according to the method described by Monreal and Reese (1968). Chitin flakes (10 g) were added into 100 ml 85% (v/v) phosphoric acid. The suspension was left stirring constantly overnight at 4°C. An excess volume of distilled water was added into the suspension to precipitate the acid-treated chitin. The precipitate was harvested by centrifugation at 10,000 rpm at 4°C for 30 min. The precipitate was washed thoroughly until the pH of the suspension was nearly neutral. The swollen chitin was kept at 4°C until used.

2.2.2.3 Effect of Types of Chitin on Chitinase Expression

The cell culture of *V. alginolyticus* was grown in VCM medium, pH 8.5 at 30°C. Chitinase activity was determined every day until the eighth day of incubation. Chitinase activity was determined when the cells were grown in the medium with or without swollen chitin and flake chitin.

2.2.2.4 Effect of Time Course on Chitinase Expression

To investigate the chitinase activity from the marine *Vibrio*, single colonies of each *Vibrio* were picked and transferred to 10 ml of appropriate medium containing 5% (w/v) swollen chitin. The cell culture was incubated at conditions mentioned previously. One ml of the culture was taken every day for seven days in order to determine the chitinase activity and cell density (OD₆₆₀) as well as protein concentration.

2.2.2.5 Expression of Chitinase from Marine *Vibrio* on Chitin Plates

Single colonies of marine *Vibrio* except *V. marinus*, and *V. splendidus* were picked and transferred to 5 ml of marine medium and grown overnight at 30°C with 200 rpm shaking. The cell culture was stabbed into marine agar plates containing 1% (w/v) swollen chitin using a sterile toothpick. The plates were then incubated at 30°C for 3 days, then stored in the fridge for about 4 weeks. The clear zone around the stabbed sites was observed.

2.2.3 Attempts to Develop a Purification Scheme for Chitinase from *V. alginolyticus*

2.2.3.1 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was tested for its possibility to use for chitinase purification. The crude enzyme of *V. alginolyticus* was not prepared from fresh culture in this case, but it was obtained by combining the growth media of the cells grown from 2 to 7 days for studies of effect of chitin and effect of time on chitinase expression. The pooled culture (114 ml) was fractionated with ammonium sulphate in 2 steps of 0-35% and 35-70% saturation. Solid ammonium sulphate (234 g) was added slowly with stirring, and further stirred overnight at 4°C. The precipitate obtained from 0-35% saturation was harvested by centrifugation at 10,000 rpm for 50 min at 4°C. Ammonium sulphate (29 g) was added into 0-35% supernatant to reach 70% saturation, then further stirred overnight at 4°C. The 35-70% precipitate was obtained by centrifugation at 10,000 rpm for 30 min at 4°C. Fractions of 0-35% precipitate and 35-70% precipitate were redissolved in small volumes of 10 mM potassium phosphate buffer, pH 7.0, then dialysed overnight against 1000 ml of the same buffer with three changes. The three fractions of 0-35% precipitate, and 35-70% precipitate and 35-70% supernatant were assayed for chitinase activity as well as for protein content. Specific activity and yield obtained from each fraction were determined.

2.2.3.2 Chromatography on DEAE-Sephadex A-50

Five ml of 35-70% precipitate (43 mg) obtained from ammonium sulphate fractionation was applied to a DEAE Sephadex A-50 column (2 cm x 8 cm) pre-equilibrated in 10 mM potassium phosphate, pH 7.0. The column was washed with 3 volumes of the same buffer before a gradient of 0-0.5 M sodium chloride prepared in the starting buffer (eight column volumes) was applied. The column elution was performed at 4°C at a constant flow rate of 30 ml/h. Fractions (2 ml) were collected and assayed for chitinase activity as well as A_{280} . The ionic strength of every fifth fractions was determined.

2.2.3.3 Chitin Affinity Chromatography

The chitin affinity step was based on the method described by Molano *et al.* (1979). The dialysed pooled 35-70% saturation precipitate obtained from the ammonium sulphate step was applied to a chitin column (2 cm x 10 cm) pre-equilibrated with 10 mM potassium buffer, pH 7.0. After the column was percolated for 1 h, it was washed with the same buffer until no absorbance at 280 nm was detected, then different elution conditions were tested including 5% (v/v) acetic acid, 20% (v/v) acetic acid, and 0.1 M *N*-acetylglucosamine in 10 mM potassium phosphate, pH 7.0. The chromatography was performed at a constant flow rate of 30 ml/h. Fractions (2.0 ml) were collected. Chitinase activity was determined every second fractions as well as A_{280} . The affinity chromatography was also performed using 6 M Guanidine HCl as elution agent. In this case, the column was washed with 50 ml of 0.1 M sodium carbonate buffer, pH 8.5, followed by 50 ml of 0.1 M sodium acetate buffer, pH 5.5 before 25 ml of 6 M Guanidine HCl was applied.

2.2.4 Purification of a Chitinase from *Vibrio carchariae*

2.2.4.1 Effect of Amount of Chitin on Chitinase Expression

A single colony of *V. carchariae* was picked from a streaked plate, and transferred to 10 ml marine medium, pH 7.6. The cells were cultured overnight at 30°C with 200 rpm shaking, then 1 ml samples were transferred to 6 conical tubes containing 20 ml of the same medium and variable amount of swollen chitin; 0%, 0.5% (w/v), 1.0% (w/v), 2.5% (w/v), 5% (w/v), and 10% (w/v). The cell cultures were further incubated for 48 h at 30°C. The chitinase activity was examined in each fraction as well as protein content.

2.2.4.2 Effect of Concentrations of Guanidine HCl on Release of Chitinase from Chitin Affinity

An experiment was performed in 12 microfuge tubes. Each tube contained 1.0 ml concentrated growth medium of *V. carchariae*, and 0.1 g swollen chitin. After vortex mixing, affinity binding of chitinase to chitin was allowed to proceed rapidly in 1 min, then centrifuged at maximum speed of bench top centrifuge for 1 min at room temperature. The medium was discarded, and variable concentrations of Guanidine HCl: 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, 1 M, 2 M, 3 M, 4 M, 5 M, and 6 M, were added into separate tubes, then vortexed for 1 min. The supernatants of each reaction were collected by centrifugation, and assayed for chitinase activity.

2.2.4.3 A Complete Purification of *V. carchariae* Chitinase

A single colony of *V. carchariae* was picked and subcultured overnight at 30°C in 10 ml marine broth with 200 rpm shaking. Five ml samples were transferred to two flasks of 1000 ml marine medium containing 2.5% (w/v) swollen chitin. The cells were further cultured overnight (approx. 20 h), then the cells and chitin were removed by centrifugation at 4,000 rpm, 30 min, 4°C. The supernatants (fraction GM, growth medium) were collected as two 950 ml samples and used as crude material for chitinase purification. Purification steps were performed at room temperature unless stated otherwise as follows:

1. Swollen chitin (25 g) was added to each sample to bind the secreted chitinase.
2. The suspensions were stirred at 4°C for 5 min, then centrifuged for 20 min (4,000 rpm, 4°C).
3. The step was repeated again in order to complete binding of chitinase to the chitin affinity. Then the samples of precipitated chitin and bound protein were combined, and washed by centrifugation with 0.1 M sodium carbonate buffer, pH 8.5 until the A_{280} was less than 0.1.

4. The samples were further washed by centrifugation with 0.1 M sodium acetate buffer, pH 5.5 until A_{280} was approx. zero.
5. Guanidine HCl (2 M, 25 ml) was added and the suspension was stirred at 4°C for 2 min, then left overnight at room temperature to release bound chitinase.
6. The chitin was removed by centrifugation (12,000 rpm, 15 min, 4°C), the elution step was repeated again with the same volume of 2 M Guanidine HCl.
7. The guanidine-eluted protein solutions obtained from two fractions were combined, then dialysed extensively overnight against 20 mM potassium phosphate, pH 7.0 at 4°C (fraction CA, chitin affinity).
8. After centrifugation (12,000 rpm, 15 min, 4°C) to remove undissolved materials, the CA fraction (52 ml) was concentrated by ammonium sulphate precipitation (35-70% saturation).
9. After centrifugation (12,000 rpm, 15 min, 4°C), The CA fraction (76 mg) was dissolved in 2 ml of 20 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl.
10. The solution was applied to a Sephacryl S200-HR column (2 cm x 75 cm) pre-equilibrated in 20 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl.
11. The column was run with a constant flow rate of 10 ml/h maintained.
12. Fractions (2.5 ml) were collected. The chitinase activity and A_{280} were determined every second fractions.
13. Chitinase containing fractions (f_{31-33} and f_{34-40}), were pooled separately (fraction S200, Sephacryl S200 HR filtration).
14. The S200 fraction (pooled from fractions 34-40, 8 mg) was dialysed extensively overnight against 20 mM potassium phosphate, pH 7.0 to remove NaCl.
15. The solution was centrifuged 5 min at maximum speed of bench top centrifuge to remove undissolved materials, then applied to a MonoQ column (0.5 cm x 5 cm) using a Pharmacia FPLC[®] system. The column was pre-equilibrated with 20 mM potassium phosphate, pH 7.0, and every solutions were filtered through a Millipore GS membrane (2 μ M) before used.

16. A constant flow rate of 1.0 ml/min and a constant pressure of 1.5 mPa were maintained throughout of the run. Chart speed of 0.5 cm/min was used.
17. The column was accomplished in 8 cycles. Each cycle, 2.25 ml of the S200 fraction was injected, then washed with 10 ml of the equilibrating buffer.
18. After washing step, a gradient of 0-70% (w/v) NaCl dissolved in the equilibrating buffer was applied.
19. Fractions (0.5 ml), where the protein profile was observed, were collected and assayed for chitinase as well as A_{280} .
20. The chitinase containing fractions were analysed on SDS/PAGE, and stored at -20°C.

2.2.5 Determination of Protein Concentration

Protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as a standard (0.2-20 μ g). The assay mixture contained 20 μ l of diluted sample, 50 μ l of 1M NaOH, and 1.0 ml of dye reagent. After mixing and incubating for 5 min, the absorbance was measured at 595 nm using Hitachi U-1100 spectrophotometer.

In the case of chromatographic separations, protein concentration was monitored by measuring UV absorption at 280 nm.

2.2.6 Determination of the Subunit Molecular Weight of Chitinase SDS/PAGE

Denaturing gel electrophoresis was performed according to the method of Laemmli (1970). The separating gel (9 cm x 10 cm x 0.75 cm) contained 10% polyacrylamide and the stacking gel contained 5% acrylamide. Three parts of samples after being mixed with one part of sample buffer was boiled at 100°C for 3 min, then applied onto the gel. Electrophoresis was carried out with a constant current of 30 mA until the tracking dye reached the bottom of the gel, at which time the electrophoresis was

stopped. The gel was Coomassie stained for half an hour at room temperature, then destained with destaining solution until excess of dye was removed.

The molecular masses of protein bands were calculated using low molecular mass markers (phosphorylase b (97kDa), bovine serum albumin (66kDa), ovalbumin (45 kDa), glyceraldehyde-3-D-dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), and bovine pancreatic trypsinogen (24 kDa)).

2.2.7 Determination of Chitinase Activity under Non-denaturing Gel Electrophoresis

Non-denaturing gel electrophoresis was performed by a system modified from Hames (1990). The separating gel contained 10% acrylamide, and the stacking gel contained 5% acrylamide. Three parts of sample were mixed with one part of sample buffer before being applied directly onto the gel. Electrophoresis was carried out at 4°C under a constant current of 30 mA for 90 min. The gel was stained for chitinase activity by incubating a small volume of 1mM of 4-methylumbelliferyl- β -D-N-acetylglucobioside (4-MU-[GlcNAc]₂) or 1 mM of 4-methylumbelliferyl-N,N',N'',N'''- β -D-acytlyglucotetraoside (4-MU-[GlcNAc]₄) solution prepared in 20 mM potassium phosphate buffer, pH 7.0. The gel was incubated in the substrate solution in the dark at room temperature for 5 min with gentle shaking, then the bright purplish fluorescent bands of methylumbelliferone released were viewed under ultraviolet light on a 312 nm transilluminator.

2.2.8 Determination of Native Molecular Mass of Chitinase

To determine the native molecular mass, one ml of concentrated CA fraction containing 5 mg protein was applied to the same Sephacryl S200-HR column. The chromatography was performed in the same manner as described previously in section 2.2.4.3. The molecular mass of native chitinase was determined from the calibration curve when the same column was calibrated with 2-3 mg of standard proteins

included phosphorelase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and glyceraldehyde-3-D-dehydrogenase (36 kDa).

2.2.9 Carbohydrate Analysis after Chitin Affinity

2.2.9.1 Release of Carbohydrate from Affinity-Purified Chitinase

One ml samples of CA fraction prepared freshly from 2,000 ml culture were aliquoted into five microfuge tubes, which were incubated separately at 37°C at different times of 1h, 2h, 3h, 4h, 5h, and overnight. Samples (25 µl) from each aliquot were applied onto 12% SDS/PAGE gel to investigate change of protein pattern due to a result of release of carbohydrate from the chitinase molecules compared with when the protein was not incubated.

2.2.9.2 Sample Preparation for Carbohydrate Analysis

Paper chromatography and paper electrophoresis were used to investigate qualitatively the presence of carbohydrate bound by the chitinase molecules. Samples were prepared by incubating 1.0 ml of freshly prepared CA fractions obtained as mentioned in section 2.2.9.1. The protein solutions were hydrolysed by addition of 1 ml 4 M trifluoroacetic acid (TFA). After mixing, one ml of the solution was transferred into a screwed cap tube, and heated in the heating block at 120°C for 2 h. After heating, the solution was centrifuged at 4,000 rpm at room temperature for 5 min to remove undissolved materials. The clear solution was transferred to a new glass tube, and it was cool-dried in vacuo. The dried sample was mixed vigorously with 0.5 ml freshly prepared acetic anhydride/pyridine (1:1 by vol.). The solution was then transferred to a new screwed cap tube, and further heated at 100°C in the heating block for 1 h. The acetylated solution was transferred to a new glass tube, and cool dried in vacuo again. The dried sample was re-hydrolysed in 4 M TFA as mentioned at the beginning of the paragraph. After acid hydrolysis, the sample was cool-dried in vacuo, and then the dried sample was resuspended in 300 µl dH₂O and ready for

applying onto paper chromatography or paper electrophoresis. Paper chromatography and paper electrophoresis were performed according to the method described by Fry (1988) as detailed in the following sections.

2.2.9.3 Paper Chromatography (PC)

1. 10 μ l of each sample was loaded with drying three times as a single spot 9 cm from the short edge of a Whatman paper No 1 (46 cm x 57cm). Each spot was spaced 2.5 cm apart. 10 μ l of lysine solution was loaded at both ends of the loading position as a marker.
2. The paper with dried samples was hung with long edge vertical from a long glass trough containing butanol/acetic acid/dH₂O (12:3:5 by vol.).
3. The whole assembly was housed in a large glass tank containing a small volume of the solvent at the bottom to equilibrate the atmosphere in the tank. The tank was then closed with a greased, well fitting, and glass lid.
4. The chromatography was performed in the fume cupboard. The solvent from the trough was allowed to migrate down by capillary action for 43 h at room temperature.
5. The paper was taken out from the tank dried and stained by the silver stain method as mentioned in section 2.2.9.5

2.2.9.4 Paper Electrophoresis (PE)

1. The samples were applied in the same way as for paper chromatography but onto Whatman 3 mm paper (23 cm x 57 cm), and methylene blue was used as a marker.
2. The loaded paper was laid on a sheet of glass and wetted with buffer, pH 2 containing formic acid/acetic acid/water (1:4:45 by vol.) delivered from a pipette. The area of paper bearing the samples was raised between the glass rods and wetted last. This step was done carefully to prevent the samples running into one another.

3. Excess buffer was lightly blotted from the electrophoresis paper with dry paper tissues.
4. The paper was suspended in a large tank filled with immiscible coolant (white spirit). The top of the paper is held in a trough containing about 250 ml of buffer and a platinum cathode; the bottom dips into another layer of buffer, about 500 ml, containing a platinum anode.
5. High-voltage was applied with 3 kV for 1 h. The temperature was kept below 30°C by coils with running tap water.
6. After electrophoresis, the paper was taken out and hung up side down in the oven at 60°C for an hour to dry the paper.
7. The paper was silver stained as described in section 2.2.9.5

2.2.9.5 Stain for Paper Chromatograms

Silver nitrate was tested to be the most sensitive and suitable method for glucosamine detection in this experiment. The steps for staining are outlined as follows:

Reagents:

Solution A: Saturated AgNO_3 (2 ml) was added to 400 ml acetone with rapid stirring. If a precipitate appeared, it was redissolved by addition of a minimum amount of H_2O .

Solution B: Mixed 1.25 ml 10 mM NaOH with 100 ml absolute ethanol.

Both solutions were prepared freshly on day of use.

Method:

1. Chromatogram was dipped through an excess volume of solution A.
2. Air-dried for 15 min.
3. Dipped rapidly through solution B.
4. Re-dried for 15 min.
5. Repeated dipping through solution B

6. Dipped slowly through thiosulfate solution (see appendix III), then transferred straight to sink carefully.
7. Immersed in a large volume of water, then rinsed extensively with running tap water for 1 h.
8. Air-dried for 30 min.

2.2.9.6 Quantitative Analysis

Carbohydrate contents of CA fractions incubated at different times of 1h, 2h, 3 h, 4h, 5h, and overnight were determined quantitatively by PAHBAH method as described by Lever (1972). 600 μ l of each fraction was spun through vivaspin membrane with 10,000 molecular weight cut off at 4,000 rpm, 4°C for 20 min. The retentate was recovered by resuspending with 600 μ l dH₂O. The flow through was also collected. Both fractions were acid-hydrolysed as described in section 2.9.2.1. The PAHBAH reaction for reducing sugar was performed as follows:

Reagents:

Solution A: 5% (w/v) p-hydroxy benzoic acid hydrazide in 0.5 M HCl

Solution B: 10 ml solution A freshly mixed with 40 ml 0.5 M NaOH

Method: To 0.25 ml acid-hydrolysed CA retentate or flow through, 0.75 ml solution B was added. After mixing, the reaction was incubated in a boiling waterbath for 5 min. The reaction was stopped by cooling the tube quickly on ice for 5 min. The A₄₁₀ was read. The amount of reducing sugar detected was calculated using glucosamine (0.1-10 μ g) as a sugar standard.

2.2.10 Western Blotting

Western Blotting was performed according to the Enhanced Chemiluminescence (ECL) protocol as follows:

1. SDS/PAGE gels were performed in duplicate by the method outlined in section 2.2.6. After electrophoresis, one gel was Coomassie stained while the other gel was used to transfer protein bands onto Hyperbond C nitrocellulose membrane by a semi-dry procedure using a Pharmacia Multiphore II transfer system.
2. Transfer was accomplished after 1 h at a constant current of 30 mA.
3. After transference, non-specific binding sites were blocked by immersing the membrane in 5% (w/v) skimmed milk protein in Phosphate buffered saline plus 1% (v/v) Tween-20 (PBS-T) overnight at 4°C.
4. The membrane was briefly rinsed in PBS-T using two changes (50 ml each) and then once for 15 min and twice for 5 min with fresh changes of PBS-T. The membrane was shaken gently at room temperature during the steps of washing and incubation.
5. The membrane was incubated for an hour with the primary antibody, rabbit anti-chitinase serum, which was diluted 1/2000 times in PBS-T.
6. The membrane was washed as detailed in step 4.
7. The membrane was incubated for 45 min with the secondary antibody, goat anti-rabbit IgG, conjugated to horseradish peroxidase. The antibody was diluted 1/2000 times in PBS-T prior to incubation.
8. The membrane was washed again as detailed in step 4.
9. The membrane was soaked with ECL Western blotting detection (luminol) reagents for 1-2 min.
10. The detection reagents were drained and the blot was wrapped in Saran wrap.
11. The BioMax MS-1 film (20.3 cm x 25.4 cm) was placed on the top of the blot and exposed for 30 sec or 1 min.
12. The film was developed automatically using X-Ograph Compact X2 Developer.

2.2.11 Preparation of Polyclonal Antibodies from the Purified *V. carchariae* Chitinase

Antisera against purified *V. carchariae* chitinase were obtained from a female New Zealand rabbit. The procedure was carried out by the Scottish Antibody Production Unit, Law Hospital, Lanarkshire. 50 µl of purified chitinase (150 µg) obtained from the Mono Q fplc step was emulsified with 50 µl TiterMax[®]Gold adjuvant according to the instruction enclosed with the product. Blood was collected from the rabbit prior to the immunisation schedule. The antigen was injected intramuscularly into two different sites of the rabbit. Test bleeds (25 ml) were collected twice at the second and the third weeks of injection with no boosting required. The rabbit was bled by cardiac puncture after one month of immunisation. The titre of each test bleed was determined by Western blotting. The final bleed of approximately 100 ml was aliquoted in to small volumes of 10 µl, 100 µl, and 1 ml batches, and stored at -20°C.

2.2.12 Determination of Titre of Antiserum

The titre of the antiserum was tested against purified *V. carchariae* chitinase. Purified chitinase (Approx. 10 µg) was loaded into each well on SDS/PAGE gels. After electrophoresis, one track of the gel was cut and Coomassie stained, while the rest of the gel was analysed by Western blotting. The Western blotting was performed with different dilutions of the anti-chitinase serum; 2,500, 5,000, 7,500, 10,000, and 20,000 times.

2.2.13 Determination of Specificity of Antiserum

The polyclonal antibodies raised were tested for their specificity towards the crude enzymes of different marine *Vibrio* (except *V. splendidus* and *V. marinus*). 20 µl of concentrated growth medium of each *Vibrio* containing about 50 µg protein was applied onto a SDS/PAGE gel. After electrophoresis, the gel was analysed by Western blotting with anti-chitinase serum at 1/5,000 dilution.

2.2.14 Determination of *N*-terminal Amino Acid Sequence of *V. carchariae* Chitinase

Protein sequencing was performed by Dr. Andy Cronshaw at the Welmet Sequencing Unit, Department of Biochemistry, University of Edinburgh, to investigate the *N*-terminal amino acid sequence of *V. carchariae* chitinase. The sample was prepared by concentrating in a Speedvac concentrator to a volume of 100 μ l (43 nmol) from 400 μ l of a Mono Q fraction that showed a single band of 63 kDa on SDS/PAGE. The concentrated sample was centrifuged at maximum speed of the bench top centrifuge to remove undissolved materials before being subjected to automatic sequencing on an Applied Biosystem 477A microsequencer with a 120A on-line phenylthiohydantoin analyser. Polybrene (2mg) was loaded onto a paper fibre filter disc which was then pre-cycled three times before being loaded with approximately 40 nmol of purified *V. carchariae* chitinase. The Edman degradation was carried out and after that, the anilinothiazolinone derivatives cleaved from the protein were converted automatically into the more stable phenylthiohydantoin forms and separated on an Applied Biosystems PTH C₁₈ (5 nm particle size; 2.1 nm x 220 nm) column that was eluted with a 0-100% (v/v) linear gradient of acetonitrile. The gradient was formed with an aqueous 5% (v/v) solution of tetrahydrofuran as solvent A and acetonitrile as solvent B. Chromatography was performed at 55°C and the amino acid profile was monitored at 269 nm. Twenty amino acid residues were sequenced.

2.2.15 Agarose Gel Electrophoresis

1% (w/v) agarose gel was melted in 60 ml of Tris.Borate-EDTA (TBE) buffer, pH 8.0, and ethidium bromide was added to a final concentration of 0.5 μ g/ml in order to visualise the DNA by UV light. The molten gel was allowed to cool down for a few minutes at room temperature, then cast in a Pharmacia mini-gel system. One part of DNA samples was mixed with 5 parts of 6 x concentrated gel loading buffer, prior to loading into each well alongside the standard λ DNA restricted with *Hind* III, or 1 kb ladder DNA marker (250 ng of total DNA). A constant voltage of 100 V was applied

to the electrophoresis tank until the bromophenol blue migrated the appropriate distance through the gel. DNA bands were then examined under UV light on a 312 nm transilluminator.

2.2.16 Plasmid DNA Preparation

2.2.16.1 Small Scale Preparation

Plasmid DNA was prepared on a small scale according to the standard method described by Maniatis *et al.* (1982). The procedure in details consisted of the following steps:

1. A single colony was picked and transferred to 5 ml of Luria Broth medium (LB) culture containing 100 µg/ml ampicillin and grown overnight at 37°C with 200 rpm shaking.
2. The cell pellets were harvested by centrifugation at 4,000 rpm for 15 min at 4°C.
3. After inverting the universal bottle for a few minutes to remove some traces of the media, the pellet was resuspended in 100 µl ice-cold solution I containing 50 mM Tris.HCl, pH 8.0 and 10 mM EDTA with vigorous vortexing.
4. 200 µl of freshly prepared solution II containing 200 mM NaOH, and 1% (w/v) SDS was then added into the cell suspension. The solution was mixed by inverting the bottle slowly for five times.
5. 150 µl of ice-cold solution III containing 3.0 M potassium acetate, pH 5.5 was added into the suspension, and the bottle was inverted gently for 5 times, then the bottle was stored on ice for 5 min.
6. The cell lysate was transferred to a microfuge tube, then centrifuged at 10,000 rpm for 30 min, 4°C.
7. The supernatant was transferred to a new microfuge tube. The DNA in the cell suspension was purified by addition of an equal volume of phenol/chloroform/isoamyl ethanol (25:24:1 by vol.). The solution was vortexed

- vigorously for 1 min, then centrifuged at maximum speed (13,000 rpm) of the bench top centrifuge for 5 min at room temperature.
8. The upper phase solution was transferred to a new microfuge tube, and the step7 was repeated again.
 9. The solution was added to 2.5 volumes of ice-cold absolute ethanol, mixed by vortexing, then left standing at -20°C for 30 min.
 10. The DNA precipitate was collected by centrifugation at a maximum speed with the bench top centrifuge for 5 min at 4°C.
 11. The DNA pellet was washed with 500 µl of ice-cold 70% (v/v) ethanol, then air-dried for 30 min or until the pellet became white.
 12. The dried DNA pellet was resuspended with 50 µl TE buffer containing 50 µg/ml RNase.
 13. Purity of DNA was determined by measuring A_{260} relatively to A_{280} . The DNA in the solution was quantitated either by spectrophotometry (A_{260}) or an agarose gel.

2.2.16.2 Large Scale Preparation of Plasmid DNA

Plasmid DNA was prepared on a large scale and purified using the QIAGEN Maxi kit according to the protocol supplied with the product as detailed below:

1. A single colony was picked from a freshly streaked LB medium containing 100 µg/ml ampicillin (LB/amp) agar plate and inoculated a starter culture of 5 ml LB/amp media. The culture was incubated for 8 h at 37°C with 200 rpm shaking.
2. The starter culture was transferred into 100 ml of the same medium, and grown at 37°C for 16 h with 200 rpm shaking.
3. The bacterial cells were harvested by centrifugation at 10,000 rpm for 15 min at 4°C. Bacterial pellet was resuspended in 10 ml buffer "P1" containing 50 mM Tris.HCl, pH 8.0, 10 mM EDTA, 100 µg/ml Rnase.
4. The cell suspension was added with lysis buffer "P2" containing 200 mM NaOH, and 1% SDS (10 ml), and mixed gently but thoroughly by inverting 4-6 times, then incubated at room temperature for 5 min.

5. The cell suspension was added with chilled neutralisation buffer "P3" containing 3.0 M potassium acetate, pH 5.5 (10 ml), and mixed immediately but gently by inverting 4-6 times, then incubated on ice for 20 min.
6. The supernatant containing plasmid DNA was removed promptly after centrifugation at 12,000 rpm for 30 min at 4°C.
7. The supernatant was re-centrifuged at 12,000 rpm for 15 min at 4°C, then supernatant containing plasmid DNA was removed promptly.
8. A QIAGEN-tip 500 was equilibrated by applying 10 ml equilibration buffer "QBT" containing 750 mM NaCl, 50 mM MOPS, pH 7.0, and 15% (v/v) isopropanol, and the column was allowed to empty by gravity flow.
9. Sample from step 8 was applied to the QIAGEN-tip and allowed to enter the resin by gravity flow.
10. The QIAGEN-tip was washed with 2 x 30 ml wash buffer "QC" containing 1.0 M NaCl, 50 mM MOPS, pH 7.0, and 15% (v/v) isopropanol.
11. DNA was eluted with 15 ml elution buffer "QF" containing 1.25 mM NaCl, 50 mM Tris.HCl, pH 8.5, and 15% (v/v) isopropanol.
12. DNA was precipitated by adding 10.5 ml (0.7 volumes) room temperature isopropanol to the eluted DNA. The DNA solution was mixed and centrifuged immediately at 12,000 rpm for 30 min at 4°C. Supernatant was decanted carefully.
13. DNA pellet was washed with 5 ml of room temperature 70% (v/v) ethanol, and centrifuged at 12,000 rpm for 10 min. Supernatant was decanted carefully without disturbing the pellet.
14. The DNA pellet was air-dried for 5-10 min, and redissolved in a suitable volume of Tris.HCl-EDTA (TE) buffer, pH 8.0.
15. Purity of DNA was determined by measuring A_{260} relatively to A_{280} . The DNA in the solution was quantitated either by spectrophotometer (A_{260}) or an agarose gel.

2.2.17 Bacterial Transformation

2.2.17.1 Preparation of Competent Cells

Competent *E. coli* type cells strain XL1 blue were prepared for electroporation by the method provided by Dr. J F Elliott, Department of Microbiology and Immunology, Stanford University, Palo Alto, California. The steps are detailed as follows:

1. Two 0.5 ml samples of stock culture stored at -70°C were subcultured into 50 ml LB in two 250 ml-flasks. The cell cultures were incubated overnight at 37°C with 200 rpm shaking.
2. The cell cultures were transferred into 1,000 ml of LB in two 2-litre flasks and further incubated for approximately 3h. OD_{600} was checked every hour until the OD reached 0.6-0.7.
3. The flasks were chilled on ice for 5 min, then the cultures were transferred to two 1-litre centrifuge bottles, and centrifuged at 4,000 rpm for 35 min at 4°C .
4. The media were poured off immediately and the tubes were inverted for a few minutes to drain the last traces of media.
5. Each pellet was resuspended in 250 ml ice-cold sterile distilled water, the cell suspension from each was pooled together in a single 1-litre centrifuge tube, then centrifuged for another 15 min at 4,000 rpm, 4°C .
6. The fluid from the cell pellets was decanted, and the tube was inverted for a few minutes.
7. Steps 5 and 6 were repeated again but the pellet was resuspended with 800 ml of ice-cold water.
8. The pellet was resuspended gently in 40 ml ice-cold 10% (v/v) glycerol, then transferred to a pre-chilled 50-ml conical tube.
9. The cell suspension was spun down, and the fluid was removed.
10. The pellet was resuspended in 2 ml ice-cold 10% (v/v) glycerol, and 50 μl of the cell suspension was aliquoted into pre-chilled 1.5-ml microfuge tubes.
11. The tubes were snapped frozen in N_2 (liquid), and stored at -70°C before use.

2.2.17.2 Introduction of DNA into the Competent *E. coli* Cells

1. 50 μ l of competent cells were mixed with ice-cold 5 μ l plasmid DNA resuspended in distilled water. The step should be handled gently to avoid air bubbles that might cause exploding while the transformation step was performed.
2. The reaction mixture was transferred to a pre-chilled metal cuvette, and then the cuvette was put into the chamber of an Hybaid Electroporator machine.
3. A quick electrical pulse of 1,800 V was applied, and 950 μ l of ice-cold SOC medium was added immediately after the pulse to prevent cell damage.
4. The cell suspension was mixed gently, then transferred to a 15-ml conical tube and incubated at 37°C for 45 min with 200 rpm shaking.
5. 50 or 100 μ l of the cell suspension was plated out on a LB agar plate containing 100 μ g/ml ampicillin, 0.5 mM IPTG, 40 μ g/ml X-Gal (LB/amp/IPTG/X-Gal), then the plate was incubated overnight at 37°C.

2.2.18 Polymerase Chain Reaction for Genomic DNA Library Screening

2.2.18.1 Design of Oligonucleotide Primers

To perform PCR reactions, three sets of degenerate oligonucleotide primers were designed from the *N*-terminus of the *V. carchariae* chitinase using the computer program called "BACKTRANSLATE". They were designated P1, P2, and P3 (see Figure below). Universal primers; M13 (5'-TGTAACGACGGCCAGT-3'), M13(rev) (5'-CAGGAAACAGCTATGACC-3'), or SP6 (5'-ATTAGGTGACAC TATA-3') were used as flanking oligonucleotide primers.

Target *N*-terminal Amino Acid Sequence

N-----**M A P T A P** S D P **V Y G S / M N A L** Q A L D R / P / I -----C
 region1 region2

Degenerate oligonucleotide primers, designated "P1", were designed from the *N*-terminal amino acid at region 1 (MAPTAP) and contained eighteen sequences (5'-ATG GC(AGT) CCG AC(CT) GC(TAG) CCG-3'). The other two degenerate oligonucleotide primers, designated "P2" and "P3", were designed from the *N*-terminal amino acid at region 2 (VYGS/MNAL). Both P2 and P3 contained 36 oligonucleotide sequences 5'-GT(TAG) TA(CT) GG(TC) III* AAC GC(TAG) CT-3' and 5'- GT (TAG)TA(TC) GG(TC) ATG AAC GC(TAG) CT-3', respectively. (I* represents Inosine)

2.2.18.2 Isolation of Genomic DNA from *V. carchariae*

Genomic DNA of *V. carchariae* was isolated according to the standard method described in Current Protocols in Molecular Biology (Ausubel *et al.*, 1994) as detailed below:

1. A hundred ml of *V. carchariae* cell culture was grown to stationary phase.
2. The cells were pelleted by centrifugation at 4,000 rpm, at 4°C for 20 min, then supernatant was discarded.
3. The cells were resuspended gently in 9.5 ml TE buffer, then 0.5 ml of 10 % (w/v) SDS and 50 µl of 20 mg/ml proteinase K was added and mixed thoroughly. The cell suspension was incubated for 1h at 37°C.
4. The cell suspension was added with 1.8 ml of 5 M NaCl, and mixed thoroughly.
5. The cell suspension was added 1.5 ml cetyltrimethylammonium bromide (CTAB)/NaCl solution, then mixed thoroughly and incubated 20 min at 65°C.
6. An equal volume of chloroform/isoamyl alcohol (24:1 by vol.) was added, then the genomic DNA was extracted thoroughly. The DNA suspension was spun 10 min at 6,000 rpm at 4°C to separate the phases.
7. Aqueous supernatant was transferred to a fresh tube using a wide-bore pipette.
8. Isopropanol (0.6 volumes) was added into the supernatant, and mixed until a stringy white DNA pellet precipitated out of solution and condensed into a tight mass.

9. The precipitate was transferred to 1 ml of 70% (v/v) ethanol in a fresh tube, by hooking it on the end of a Pasteur pipette that had been bent and sealed in a Bunsen flame.
10. The pellet was spun for 5 min at 10,000 rpm, then supernatant was removed. The pellet was redissolved in 4 ml TE buffer. The DNA was allowed to dissolve overnight at 4°C.
11. The purity of DNA was measured by spectrophotometry (A_{260}/A_{280}) and the concentration of DNA was quantitated by A_{260} as well as by an agarose gel.

2.2.18.3 Preparation of DNA Template

DNA template was prepared by digesting genomic DNA of *V. carchariae* with *EcoR* I then ligated into *EcoR* I sites of pBluescript II KS(-) vector. The digestion of pBluescript with *EcoR* I and the ligation reaction is shown below:

Digestion reactions;

For genomic DNA

2 µg genomic DNA	100 µl
80 U <i>EcoR</i> I, 40 U/µl	2 µl
10 x C <i>EcoR</i> I buffer	40 µl
spermidine, 100 mM	4 µl
purified BSA, 10 mg/ml	4 µl
distilled water to volume	400 µl

The reaction mixture was incubated overnight at 37°C, then the reaction was stopped by heating up at 65°C for 15 min.

For pBluescript

5 µg pBluescript vector	0.5 µl
20 U <i>EcoR</i> I, 10 U/µl	2 µl

10 x C <i>EcoR</i> I buffer	2 μ l
distilled water to volume	20 μ l

The reaction was incubated 2 h at 37°C, then the following reaction was performed to remove terminal phosphate groups from the double stranded DNA:

<i>EcoR</i> I digests	20 μ l
alkaline phosphatase, 1U/ μ l	12 μ l
10 x C <i>EcoR</i> I buffer	3 μ l
distilled water to volume	50 μ l

The reaction was incubated at 37°C for 45 min, then 2 μ l of 10% (w/v) SDS was added to stop the reaction. 48 μ l of dH₂O was added to bring the total volume to 100 μ l. The DNA solution was extracted twice with phenol/chloroform/isomyl alcohol solution, then ethanol precipitated. After ethanol precipitation, The DNA pellet was dissolved in 50 μ l dH₂O and analysed on an agarose gel.

Ligation reaction:

200 ng pBluescript, <i>EcoR</i> I digested	2 μ l
800 ng genomic DNA, <i>EcoR</i> I digested	2 μ l
10 x C ligation buffer	1 μ l
1 U T ₄ DNA ligase, 1U/ μ l	1 μ l
distilled water to	10 μ l

The reaction mixture was incubated overnight at 16°C, then the reaction was stopped by incubating the reaction tube at 65°C for 15 min. The reaction was then stored at -20°C until used.

2.2.18.4 Polymerase Chain Reaction (PCR)

The first round PCR reaction was performed according to the following conditions:

Reaction mixture:

DNA template (ligated pBluescript II KS(-) plasmid)	0.5µl
10 x concentrated buffer H	5 µl
MgCl ₂ , 25 mM	5µl
4 dNTPs mix, 2 mM	1µl
degenerate primers P1, 100 µM	1 µl
flanking primers M13/M13(rev), 100 µM	1µl
Tag DNA polymerase, 20U/µl	0.5 µl
distilled water to total volume	100 µl

The PCR reactions were carried out in an Appligene Thermal Cycler model Crocodile III for 32 cycles under the following conditions:

Denaturation at 95°C, 2 min for 1 cycle

Hybridisation at 55°C, 30 sec followed by 72°C, 30 sec, and 95°C, 30 sec for 30 cycles

Extension at 55°C, 30 sec, followed by 72°C, 2 min for 1 cycle

The second round PCR was performed in the same manner as the first round but degenerate primers P2 or P3 were used.

2.2.18.5 Analysis and Purification of PCR Products on Gel Electrophoresis

After PCR reactions, the PCR products were separated and purified on agarose gel. Electrophoresis was performed according to the standard procedure outlined in

seciton 2.2.15. The DNA bands were located using a short wave UV light transilluminator for the minimum length of time to avoid damage to DNA. The bands of interest were cut out of the gel using a sterile scalpel blade and placed into a sterile microfuge tube prior to starting DNA purification using QIAEX II kit from QIAGEN. The steps are detailed below.

1. The gel slices were weighed and 3 volumes of buffer QXI was added to 1 volume of gel for DNA fragments of 100 bp to 4 kb. The gel slices were resuspended by vortexing vigorously, followed by incubating at 50°C in a heating block for 5 min.
2. The QIAEX II silica-gel particles were resuspended by vortexing for 30 sec prior to addition of 30 µl of the gel particles to the suspension.
3. After vortex mixing, the microfuge tube was incubated at 50°C for 10 min and the tube was inverted every 2 min to keep the QIAEX II in suspension.
4. The microfuge tube was centrifuged for 30 sec and the supernatant was carefully removed with a pipette, and discarded.
5. The pellet comprising the QIAEX II and bound DNA was washed with 500 µl of buffer QX I to remove the residual agarose contaminants.
6. The pellet was washed twice with ethanol-containing buffer PE to remove the residual salt contamination.
7. The pellet was air-dried for 10 to 15 min or until the pellet became white.
8. The DNA pellet was resuspended in 20 µl of TE buffer by vortexing, and incubated for 5 min.
9. The supernatant was carefully removed by pipetting into a new sterile microfuge tube.
10. Step 8 and 9 were repeated and the eluates were combined and the DNA solution was analysed on an agarose gel for the purity before being ligated into a pGEM-T vector, and before the nucleotide sequence of PCR products were analysed by manual sequencing.

2.2.18.6 Preparation of DNA Template for DNA Sequencing

The PCR products purified by QIAEX II kit were ligated into a pGEM-T vector. The ligation reaction was performed in the same manner as ligation of gDNA with pBluescript vector as described in section 2.2.18.3. The ligated DNA (molar ratio between DNA template and DNA vector of 3 to 1) was electroporated into competent *E. coli* cells type strain XL1 blue. Plasmid DNA of the clones carrying PCR products was isolated by small-scale plasmid DNA preparation. To test for success of ligation reaction, the plasmid DNA was double digested with restriction enzyme *Pst* I and *Nco* I as the reaction shown below.

***Pst* I/*Nco* I digestion**

100 ng plasmid DNA	2 µl
10 x c buffer 3	2 µl
20 U <i>Pst</i> I, 20 U/µl	1 µl
20 U <i>Nco</i> I, 20 U/µl	1 µl
distilled water to volume	20 µl

The reaction mixture was incubated at 37°C for 2h, then stopped by heating up to 65°C for 15 min. 10 µl of the reaction mixture was used to analyse on an agarose gel.

2.2.18.7 Sequence Analysis of PCR Products

The sequence of PCR products was investigated by manual sequencing. The sequencing was performed according to the protocol provided with the sequencing kit as detailed below.

1. The double stranded pGEM-T plasmid carrying the PCR products was purified on 1% low melting temperature agarose gel by the plasmid purification QIAGEN kit as detailed in section 2.2.18.5.

2. The annealing mixture containing 8 μ l of purified DNA (4 μ g) and 1 μ l primer in microfuge tube was heated at 100°C for 3 min, and quick-chilled on ice for 5 min.
3. The annealing mixture was centrifuged briefly to collect the condensation prior to addition of 9.25 μ l of the labelling solution containing 1.25 μ l DMSO, 2.5 μ l of 10 x concentrated sequenase buffer, 1 μ l DTT, 2 μ l of 1/5 diluted labelling mix, 1 μ l [35 S]ATP, and 2 μ l of 1/8 diluted sequenase.
4. After the annealing mixture was incubated at room temperature for 5 min, 3.5 μ l of the mixture was transferred to 2.5 μ l of each pre-warmed termination mix (G, C, A, and T). Each termination mix consisted of 0.25 μ l DMSO and 1.25 μ l termination mix containing 10 mM ddG, ddC, ddA, or ddT.
5. The termination reactions were further incubated for another 5 min at 37°C before the reaction was stopped by addition of 4 μ l of the stop mix (95% (v/v) formamide, 20 mM EDTA, and 0.05% bromophenol blue).
6. The reactions were heated to 85°C for 5 min immediately to denature the double stranded DNA before 2.5 μ l of each reaction was loaded onto the sequencing gel. The remaining reaction was stored at -20°C for further loading.
7. The sequencing reaction was applied into the wells in the order of A,C,G, and T. A range of current from 40-45 mA was applied in order to maintain the running temperature at 55°C until the second marker dye was migrated about three quarters of the way down the gel, then the run was stopped.
8. After the end of the run, the gel plates were separated using a spatula, and the gel remaining on at one plate was fixed by soaking with sufficient volume of fixing solution containing 50% (v/v) methanol, and 10% (v/v) acetic acid for 10 min.
9. After fixing, the gel was covered with Whatman 3 MM paper, which was precisely cut to the dimensions of the gel and then dried in a vacuum drier for approximately 90 min at 75°C.
10. Autoradiography was performed using Amersham Hyper film in a Trimax 3M film cassette for 16-24 h depending on the strength of the signal. The film was then developed automatically in a X-Ograph Compact X2 developer.

2.2.19 Preparation of Genomic DNA Library from *Vibrio carchariae*

2.2.19.1 Preparation of *Sau*3A I partial digests

The genomic DNA library was prepared according to the standard method obtained from Promega manual. Genomic DNA of *V. carchariae* was isolated and partially digested with *Sau*3A I as follows:

***Sua*3A I digestion of genomic DNA**

100 µg genomic DNA	360 µl
10x concentrated <i>Sau</i> 3A I buffer	450 µl
1 mg/ml acetylated BSA	500 µl
distilled water to volume	4,500 µl

Fifty microlitres of the assay reaction was aliquoted into 10 tubes, and 50 µl of *Sau*3A I (250 mU) was added. The reaction mixture was incubated at 37°C for 30 min, then stopped by addition of 10 µl of 0.5 M EDTA. After that, the solution in each aliquot was pooled and DNA was ethanol precipitated. After precipitation, the DNA pellet was resuspended in a small volume of 200 µl. The concentrated DNA was then added with 40 µl of DNA gel loading buffer before loading onto a big well of 1% low melting temperature agarose gel. Electrophoresis was performed in the same manner as described previously but at 4°C. After electrophoresis, DNA bands between 4 -7 kb were cut under UV light, and the DNA fragments were purified by GELase as described by the method provided with the products. The DNA fragments were resuspended into a small volume of 20 µl, and the concentration of the DNA in the solution was estimated either by spectrophotometry (A_{260}/A_{280}) and the concentration of DNA was quantitated by A_{260} as well as by an agarose gel.

2.2.19.2 Preparation of pBluescript II KS(-) Vector for Ligation

pBluescript vector was digested with *Bam*H I as follows:

***Bam*H I digestion of pBluescript KS II(-) vector**

20 µg pBluescript vector	20 µl
50 U <i>Bam</i> H I, 10 U/µl	5 µl
10 x concentrated <i>Bam</i> H I buffer	2.5 µl
distilled water to volume	50 µl

The reaction mixture was incubated at 37°C for 2 h before being stopped by addition of 2 µl 0.2 M EDTA, then incubated at 70°C for 10 min. The DNA solution was ethanol precipitated, then DNA pellet was resuspended in 100 µl distilled water. 20 µl DNA gel loading buffer was added into the DNA solution, then applied onto 1% (w/v) agarose gel. Electrophoresis was performed according to the standard method outlined in section 2.2.15.

2.2.19.3 Gene Cloning

2.2.19.3.1 Small Scale Ligation

Molar ratios between DNA insert (*Sau*3A I partial digests) and DNA vector were tried as follows in order to maximise the ligation efficiency (Table 2.1).

Table 2.1 Optimisation of molar ratio of DNA insert and DNA vector.

Ligation reaction	<u>Molar ratio (insert: vector)</u>						
	1/10	1/5	1/3	1/1	3/1	5/1	10/1
Insert (ng)	50	50	50	50	50	50	300
Vector (ng)	500	250	150	50	16.7	10	30
10X C ligation buffer (µl)	2	2	2	2	2	2	4
DNA ligase (µl, 20 U/µl)	1	1	1	1	1	1	2
<i>Bam</i> H I (µl)	1	1	1	1	1	1	2

Added nuclease free water to total volume of 20 µl except molar ratio 10/1 to volume 40 µl. The reaction mixture was incubated overnight at 16°C, then the DNA was ethanol precipitated, washed, and dried as the same way as described previously. DNA pellet was resuspended in 5 µl distilled water and electroporated into competent

E. coli cells type strain XL1 Blue. 10 µl of each transformation reaction was plated out on six 90 mm round plates containing LB agar plus 100 µg/ml ampicillin, 0.5 mM IPTG, 40 µg/ml X-Gal, and 1% (w/v) swollen chitin. The plates were incubated overnight at 37°C, then white colonies were counted.

2.2.19.3.2 Large Scale Ligation

The *Sau3A* I partial digests were ligated into *BamH* I sites of pBluescript vector. Four ligation reactions were set up as follows:

Ligation of *Sau3A* I partial digests to *BamH* I cut pBluescript vector

300 ng <i>Sau3A</i> I partial digests	30 µl
50 ng pBluescript vector	0.5 µl
10 x concentrated ligation buffer	4 µl
20 units <i>BamH</i> I, 10 U/µl	2 µl
40 units T ₄ DNA ligase, 20 U/µl	2 µl
distilled water to volume	40 µl

The reaction mixture was incubated overnight at 16°C, then the DNA was ethanol precipitated, washed, and dried as the same way as described previously. DNA pellet was resuspended in 5 µl distilled water and electroporated into competent *E. coli* cells type strain XL1 Blue. 500 µl of each transformation reaction was plated out on six 140 mm round plates containing LB agar plus 100 µg/ml ampicillin, 0.5 mM IPTG, 40 µg/ml X-Gal, and 1% (w/v) swollen chitin. The plates were incubated overnight at 37°C, then white colonies were counted and screened for the clones carrying chitinase gene.

2.2.20 Screening of Genomic DNA Library

The genomic DNA library was screened by an immunological method and expression of the chitinase gene was confirmed by chitin plate assay.

2.2.20.1 Immunological Assay

Screening of genomic DNA using antibodies was performed according to Sedgwick *et al.* (1991). The primary screening was performed with colonies grown on the plates which were lifted onto dry Hyperbond C nitrocellulose membrane circles. The membranes were then placed on Whatman 3MM paper soaked with 5 % (w/v) SDS, and incubated for 30 min at 85°C to lyse and fix the cells. After that, the lifts were blocked in 5% (w/v) skimmed milk in PBS-T buffer for 30 min, and the subsequent steps followed the Western blotting protocol outlined in section 2.2.10. The plates were re-incubated for an hour to allow the cells to recover, then stored in the fridge. The secondary screening was performed by looping out twelve areas containing the clones that showed signals with anti-chitinase serum, then grown up in 5 ml LB/amp medium for about 6 h at 37°C with 200 rpm shaking. The cell cultures were diluted to obtain the density of the cells less than 10^3 cells/ml, and plated out on LB agar plates, then grown overnight at 37°C. The colonies were lifted and the screening followed the method applied for primary screening.

2.2.20.2 Chitin Plate Assay

Single colonies from each of eight clones designated P1C1, P1C2, P2C1, P2C2, P2C3, P3C1, P3C2, P4C1, that showed signals with anti-chitinase serum were picked into 5 ml LB/amp, and grown overnight at 37°C with 200 rpm shaking. Each clone was streaked on a LB/amp agar plate containing 1% (w/v) swollen chitin, then incubated at 30°C for one week, followed by another week at room temperature before being stored in the fridge. The clear zone produced around the streak site was observed.

2.2.21 Expression of the Clones Carrying Chitinase Gene

Single colonies from the clones carrying chitinase were picked into 3 ml LB/amp or LB/amp containing 1% (w/v) swollen chitin, and grown overnight at 37°C, with 200 rpm shaking. 100 µl of the cell culture was taken and centrifuged at 4,000 rpm for 20

min at 4°C, then the medium was collected as well as the cell pellet. The medium was dialysed against 2 x 2000 ml dH₂O overnight, then concentrated to a small volume of 50 µl by Speedvac concentrator. The pellet was mixed with 100 µl of 3 x SDS gel loading buffer, then transferred to a microfuge tube. The sample was boiled at 100°C for 5 min. Undissolved materials were removed by centrifugation at maximum speed of a bench top centrifuge for 5 min at room temperature. 25 µl of each concentrated sample and its cell extract were loaded separately onto 10% SDS/PAGE gel. After electrophoresis, the protein bands were transferred to Hyperbond C nitrocellulose membrane, and Western blotting was performed. Molecular size of the protein bands that reacted with anti-chitinase serum was compared with that of *V. carchariae* chitinase. The expression of chitinase was also determined when the cells were grown in the medium with and without swollen chitin added.

2.2.22 Restriction Investigation of the Clones Carrying Chitinase

2.2.22.1 *EcoR* I Digestion

Small-scale preparations of plasmid DNA of all the clones carrying DNA insert, which showed signals with anti-chitinase serum, were digested with *EcoR* I. The reaction mixture comprised:

100 ng plasmid DNA	5 µl
10 x concentrated <i>EcoR</i> I buffer	2 µl
20 U <i>EcoR</i> I, 20 U/µl	1 µl
distilled water to volume	20 µl

The reaction was incubated at 37°C for 90 min, then 5 µl of DNA gel loading buffer was added. 20 µl of the reaction mixture was taken and analysed on an agarose gel.

2.2.22.2 Restriction Maps of P1C1 and P3C1 Clones

Small-scale preparations of plasmid DNA of the two clones; P1C1 and P3C1, were digested with ten different restriction enzymes; *Kpn* I, *Sal* I, *Cla* I, *Pst* I, *Spe* I, *Xba* I,

EcoR I, *Hind* III, *Sau*3A I, and *Xho* I. The reaction was performed as described in section 2.2.22.1 but the suitable buffer was used for each enzyme. Samples obtained from each enzyme digestion were run alongside each other on agarose gels, and the restriction maps of both clones were illustrated.

2.2.23 Determination of DNA Sequence of the Clone Carrying a Chitinase Gene

2.2.23.1 Localisation of the Chitinase Gene in DNA Insert

The localisation of the chitinase gene in DNA insert was investigated by a consideration of restriction maps and expression of the recombinant chitinase on chitin plate. Small scale preparations of plasmid DNA of P3C1 were digested with three different restriction enzymes, *EcoR* I, *Sal* I, and *Cla* I (see section 2.2.22.1 for digestion reaction), The bigger fragment obtained from each enzyme was re-ligated and electroporated into the same host. The clones were designated P3C1/*EcoR* I, P3C1/*Sal* I, and P3C1/*Cla* I. The transformed culture was plated on LB/amp plate containing 1% (w/v) swollen chitin, and incubated overnight at 37°C. Western blotting was performed directly from the plate as described in section 2.2.20.1. Expression of the chitinase of the clone digested with each enzyme was observed.

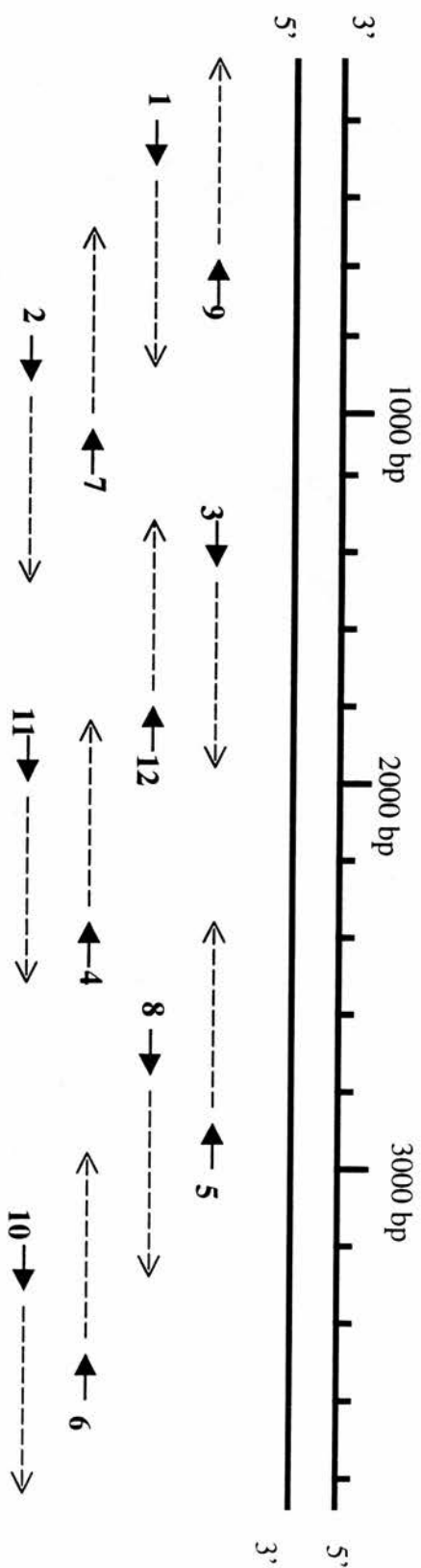
2.2.23.2 DNA Sequence of P3C1/*Cla* I Clone

To investigate the nucleotide sequence of the chitinase gene, large-scale preparation of plasmid DNA of the P3C1/*Cla* I clone was made using QIAGEN maxi kit as described in section 2.2.16.2. Double-stranded DNA was sequenced automatically by Oswel using plasmid DNA of P3C1/*Cla* I (100 µg) as template. Figure 2.1 illustrates where the oligonucleotide primers were designed along the sequence of P3C1/*Cla* I clone.

2.2.23.3 Manual Sequencing of the Upstream End of P3C1 Clone

To investigate the upstream part of the chitinase gene which was missing from the P3C1/*Cla* I clone, an oligonucleotide (5'-GTAAATTTCACTTTGATCTT-3') was

Figure 2.1 Illustration of the positions of DNA sequence of P3C1/*Cla* I clone where each oligonucleotide was designed. Number given represents order of primers that were used for determination of nucleotide sequences of each fragment. Direction of sequencing is indicated as arrows.



designed from the nucleotide sequence at a hundred bases downstream of the *Cla* I site (near 5'-end) of P3C1/*Cla* I (see Figure 2.2). Manual sequencing was performed using 8 µg plasmid DNA of P3C1 prepared by QIAGEN maxi kit as DNA template.

2.2.23.4 A complete Sequence of the Entire Chitinase Gene

After about 250 bases of upstream region of the chitinase gene were found by manual sequencing, plasmid DNA of P3C1 clone (20 µg) was sent to Oswel to determine a complete nucleotide sequence of the upstream region of the chitinase gene. The automatic sequencing was performed using the same oligonucleotide as for manual sequencing. The entire sequence of the chitinase gene was obtained by assembling the upstream part of the gene with the sequence after *Cla* I site of P3C1/*Cla* I clone. The total 4,400 bases containing the entire chitinase gene was examined for the promoter site, Shine-Dalgarno sequence, signal peptide sequence, sequence encoded *N*-terminal amino acid compared with the native chitinase, stop codon, and invert repeated sequence after the stop codon.

2.2.23.5 Computing Analysis

The computer program called "GENSTAR" was used to analyse the DNA sequence. The computer program called "MEGALIGN" was used to align the DNA sequences. The computer program called "BLASTX" was used to compare the sequence with the sequences obtained in GenBank database. The computer program in "GCG" package obtained from Seqnet was used to analyse the amino acid sequences obtained from databases. The program "CLUSTALW" was used to make the structure-based alignment for modelling. The program "O" was used to build up the modelled structure of *V. carchariae* chitinase.

2.2.24 Preliminary Studies of Recombinant Protein Expressed by P3C1 Clone

2.2.24.1 Protein Processing

Figure 2.2 The upstream sequence of the DNA fragment carried by P3C1/*Cla* I subclone. The region that was chosen to design the nucleotide primer for determination of the upstream part of P3C1 clone and the direction of sequencing are indicated as reverse arrow.

GCTCACTCATTAGGCACCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAG 69
 CGAGTGAGTAATCCGTGGGGTCCGAAATGTGAAATACGAAGGCCGAGCATACAACACACCTTAACACTC

CGGATAACAATTTACACAGGAAACAGCTATGACCATGATTACGCCAAGCGCGCAATTAACCTCTACTA 138
 GCCTATTGTTAAAGTGTGTCTTTGTGCTACTGGTACTAATGCGGTTTCGCGCTTAATTGGGAGTGAT

AAGGGAACAAAAGCTGGGTACCGGGCCCCCCTCGAGGTCGACGGTGTACGGTTCCAATAACCTTCAAT 207
 TTCCCTTGTTTTCGACCCATGGCCCGGGGGGAGCTCCAGCTGCCACATGCCAAGGTTATTGGAAGTTA

TTTCTAAAATTGAATTGGCAATGGAAACCACATCTGGCTACAACGACATGGTCAAATACCATGAACTGG 276
 AAAGATTTTAACCTAACCGTTACCTTTGGTGTAGACCGATGTTGCTGTACCAGTTTATGGTACTTGACC

CTAAGATCAAAGTGAAATTTAACCAGTGGAGTGGCACATCTGGCGACACTTACAACGTCTACTTTGACG 345
 GATTCTAGTTTCACTTTAAATTGGTCACCTCACCGTGTAGACCGCTGTGAATGTTGCAGATGAAACTGC

← upstream primer

GTGTAAAAGTGGCAACAGGCGCTATCACTGGCAGTCAAACCACAGCTTCGTTTGAATATGGTCAAGGCG 414
 CACATTTTCACCGTTGTCCGCGATAGTGACCGTCAGTTTGGTGTGCAAGCAAACCTTATACCAGTTCCGC

GCTTGTACCAAATGGAAATCGAAGCGTGTGACGCAACAGGTTGTTCTAAGAGCGCTCCGGTAGAAATTA 483
 CGAACATGGTTTACCTTTAGCTTCGCACACTGCGTTGTCCAACAAGATTCTCGCGAGGCCATCTTTAAT

CCATTGCAGATACAGACGGCTCACACTTGAAGCCTCTGACGATGAATGTTGATCCGAACAACAAGAGCT 552
 GGTAACGTCTATGTCTGCCGAGTGTGAACCTCGGAGACTGCTACTTACAACCTAGGCTTGTTGTTCTCGA

ACAACACCGATCCAAGTATCGTGATGGGTACTTATTTTGTGTAATGGGGCATCTACGGTCGTGATTACA 621
 TGTGTTGGCTAGGTTTCATAGCACTACCCATGAATAAAACAACCTACCCCGTAGATGCCAGCACTAATGT

CTGTCGACAACATGCCAGTTGATAACCTAACTCACATCCTTTACGGCTTTATCCCAATTTGTGGTCCAA 690
 GACAGCTGTTGTACGGTCAACTATTGGATTGAGTGTAGGAAATGCCGAAATAGGGTTAAACACCAGGTT

ACGAATCAGTAAAATCAGTTGGTGGTAACAGCTTTAATGCACTGCAAACGGCATGTCGTGGTGTGAATG 759
 TGCTTAGTCATTTTAGTCAACCACCATTGTGCAAAATTACGTGACGTTTGCCGTACAGCACCACACTTAC

Cell extract was prepared according to the method of Mianiaty *et al.* (1991). A single colony of P3C1 clone or *V. carchariae* from the streaked plates was picked into 100 ml of appropriate medium. The cells were grown overnight at its suitable conditions; 37°C for P3C1, and 30°C for *V. carchariae*, with 200 rpm shaking. After that, the cell pellet was harvested by centrifugation at 6,000 rpm for 20 min at 4°C, and resuspended in 10 ml 25 mM Tris.HCl buffer, pH 8.0 containing 20% (w/v) sucrose, 1 mg/ml lysozyme, and 1 mM EDTA. The cell suspension was stored on ice for 10 min, then centrifuged at 6,000 rpm for 30 min at 4°C. The supernatant was collected as the "periplasmic fraction", while the pellet was resuspended in 10 ml 20 mM Tris.HCl, pH 8.0 containing 1 mM PMSF. The cells were burst by sonicating at maximum amplitude for 4 times (30 sec each). The cells were left on ice for 1 min for each time of sonication. The cell extract was obtained at this stage. Protein processing was studied by mixing 1.0 ml freshly prepared cell extract of P3C1 with 0.5 ml of *V. carchariae*. Each extract mixture was incubated separately at 37°C for different times of 1h, 2 h, 4 h, and overnight. 20 µl of each extract mixture was loaded in duplicate alongside each other onto two SDS/PAGE gels as well as two non-denaturing PAGE gels. After electrophoresis, gels of SDS-PAGE and of non-denaturing PAGE were used for Western blotting analysis while the other gel of SDS/PAGE was Coomassie stained. Another gel of non-denaturing PAGE was stained for chitinase activity using 4-MU-[GlcNAc]₄ as substrate, followed by Coomassie staining.

2.2.24.2 Localisation of the Recombinant Chitinase

Localisation of recombinant chitinase in *E. coli* was investigated in periplasmic, cytoplasmic fractions as well as in inclusion bodies. The periplasmic fraction was prepared as described in section 2.2.24.1. Cytoplasmic fraction was obtained by collecting the supernatant after the cell extract was centrifuged at 10,000 rpm 30 min at 4°C. The inclusion bodies were obtained in the precipitate. Each fraction was loaded onto two SDS/PAGE gels as well as non-denaturing PAGE gels. After electrophoresis, each gel of SDS/PAGE and non-denaturing PAGE was analysed by Western blotting while the other two gels were Coomassie stained.

Chapter 3

Expression of Chitinases in Marine Bacteria, *Vibrio*

3.1 Introduction

Chitinases have been studied from a variety of organisms especially plants, fungi, and bacteria depending up on the purpose of study and applications. In plants, most studies have been aimed toward the development of transgenic plants that carry chitinase activity for resistance against fungal pathogens. Some higher plant chitinases have anti-fungal chitinase and/or anti-bacterial lysozyme activities (Ohishi *et al.*, 1989; and Audi *et al.*, 1990) while chitinase from microorganisms does not have such properties. Consequently, plant chitinases are also potentially useful for food preservatives.

In fungi, chitinases have been proposed to play a morphogenetic role during apical growth, cell division, and differentiation as well as a nutritional role related to saprophytic and mycoparasitic organisms in fungi (Papavizas, 1985; Cabib, 1987; and Kuranda and Robbins, 1991). Chitinases from fungi such as soil borne fungi of the genus *Trichoderma* have also been described as the best-known biological control agents against fungal plants pathogens (Papavizas, 1985; and Chet, 1987).

In bacteria, chitinases have been demonstrated to be present in a variety of microorganisms, including species isolated from soil, i.e. *Serratia marcescens* (Jones *et al.*, 1986), and *Bacillus sp.* (Watanabe *et al.*, 1990; and Trachuk *et al.*, 1996), fresh water and marine environments, i.e. *Aeromonas sp.* (Wiwat *et al.*, 1996; Huang and Su, 1996; and Hiraki *et al.*, 1997), *Alteromonas sp.* (Tsujibo *et al.*, 1992), and *Vibrio sp.* (Takahashi *et al.*, 1993). It was reported that bacteria produce chitinases to digest

chitin and utilise it primarily as carbon and energy sources. Moreover, by utilisation of chitin-containing nutrient substrates, extracellular chitinases extend the adaptability of microorganisms.

Marine bacteria, *Vibrios*, have been suggested as potential source of chitinases, and the enzyme from *Vibrio* sources seems to play an important role in the bioconversion process by converting insoluble chitin into biologically usable forms (Yu *et al.*, 1991).

In this chapter, experiments to develop a good assay for chitinase activity were described as well as screening investigations for chitinases from fourteen marine *Vibrio* species in order to obtain a suitable source of bacterium that contained a high activity of chitinase for further studies.

3.2 Evaluation of Assay Methods for Chitinase Activity

Three different methods; radioactive assay using [^{14}C]chitin, viscometric assay using glycol-chitin, and colorimetric assay using chitin-azure, were used to determine the activity of a commercial chitinase from *Serratia marcescens*. The results showed dramatic differences in the capability of the enzyme in degrading different substrates.

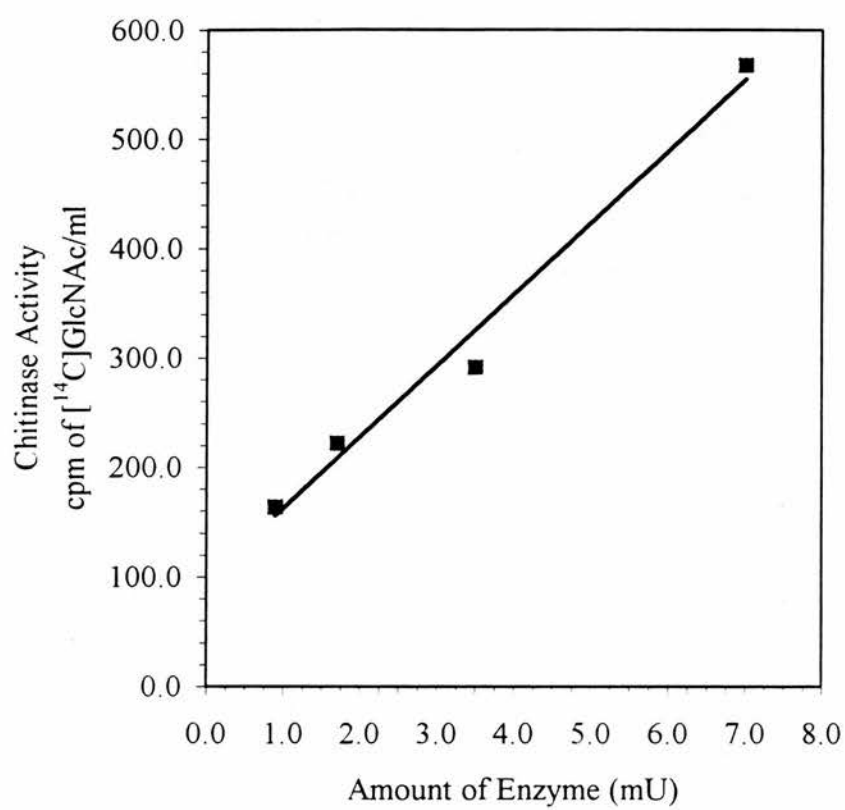
3.2.1 Determination of Chitinase Activity by Radioactive Assay

Principle: Chitinase is incubated with a suspension of [^{14}C]chitin. The water soluble oligosaccharides released are separated from the insoluble chitin by centrifugation and their radioactivity are determined.

After variable amounts of chitinase were incubated with [^{14}C]chitin substrate, prepared in suspension, for 1 h at 30°C, the radioactive soluble smaller saccharides released by action of the chitinase were measured. The result in Figure 3.1 showed that degradation of the substrate was detected at 1 h of incubation. In addition, the lowest amount of the enzyme that was able to be detected, while the curve plotted

Figure 3.1 Radioactive assay of *Serratia* chitinase towards [^{14}C]chitin

The reaction mixture contained in disposable test tubes was composed of 15 μl of [^{14}C]chitin suspension (15 mg/ml), 5 μl of 1M MES buffer, pH 6.0, variable amounts of chitinase, 0.9 mU, 1.8 mU, 3.5 mU, and 7 mU, and water to complete 0.1 ml. After incubation at 30°C for 1 h with 200 rpm shaking, the reaction was terminated by addition of 0.3 ml of 10% (w/v) trichloroacetic acid, and 3.2 ml of the reaction was transferred to a microfuge tube and centrifuged at a maximum speed of bench top centrifuge for 5 min. Supernatant (0.2 ml) was taken to a scintillation vial containing 0.8 ml of distilled water and 10.0 ml of Scintillation cocktail fluid. The solution was well mixed before being counted for ^{14}C for 2 min in the Scintillation counter.



between enzyme activity and the variable amounts of the enzyme still remained linear, was 1 mU.

3.2.2 Determination of Chitinase Activity by Viscometric Assay

Principle: The assay is based on the measurement in a viscometer of the viscosity of glycol-chitin solution, which is reduced by the action of chitinase.

Chitinase activity towards glycol-chitin was determined in the viscometric assay. The glycol-chitin was prepared by acetylation of NH_3^+ groups at C_2 on the chitosan molecule. Glycol-chitosan was titrated with 10 mM NaOH to bring the pH of the solution up to 10.0. As the pK_a of an NH_3^+ group is 7.8, the volume of NaOH used to bring the pH of the solution from 5.7 to 8.9 was measured to determine the degree of acetylation. From titration curves shown in Figure 3.2A and 3.2B, the volume of NaOH used for glycol-chitin was 0.4 ml, while 2.25 ml was used for glycol-chitosan. The percentage of acetylation was therefore calculated to be 82 %.

The decrease of viscosity of the reaction mixture as a result of action of chitinase was examined at different times from 1 min to 360 min. The curve plotted between flow time against incubation time of the reaction mixture at variable amounts of the enzyme is shown in Figure 3.3. The figure showed that the rate of degradation occurred rapidly initially then remained nearly steady after 1 h. Moreover, a decrease of viscosity of the reaction mixture was detected, even though a small amount of the enzyme (2 mU) was added.

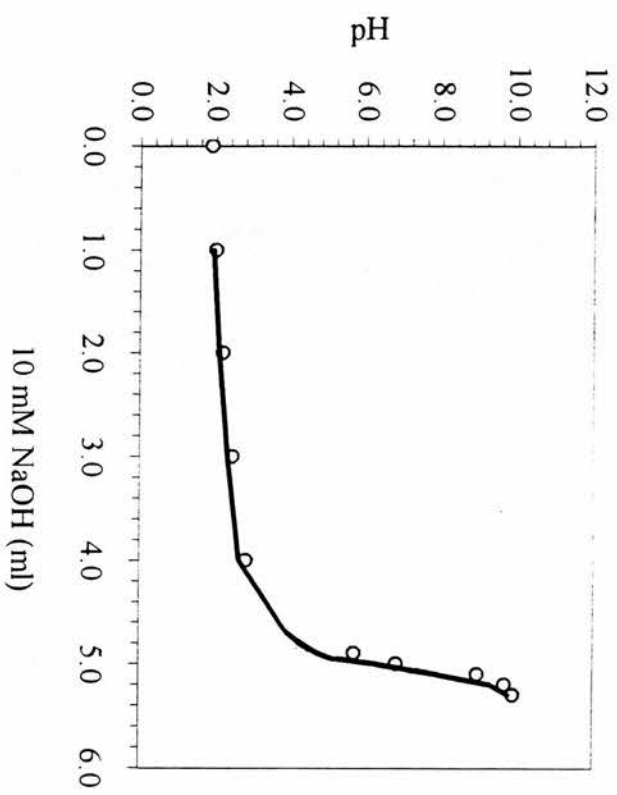
3.2.3 Determination of Chitinase Activity by Colorimetric Assay

Principle: Chitin-azure (chitin to which a blue dye molecule had been covalently attached) is incubated with the chitinase solution. The blue colour of the azure product released by action of the enzyme is measured spectrophotometrically at its λ_{max} .

Figure 3. 2 Determination of degree of acetylation of glycol-chitosan

Glycol-chitin (5.0 ml) prepared from 20 mg glycol-chitosan was titrated with 10 mM NaOH to bring the pH of the solution to 10.0. Titration of glycol-chitosan was performed in the same manner as for glycol chitin. The volumes that brought the pH of the two solutions from 5.7 to 8.9 were observed and used for determination of percentage of acetylation of glycol-chitosan. The titration curve of glycol-chitin is shown in Figure 2A, and the titration curve of glycol-chitosan is shown in Figure 2B.

A)



B)

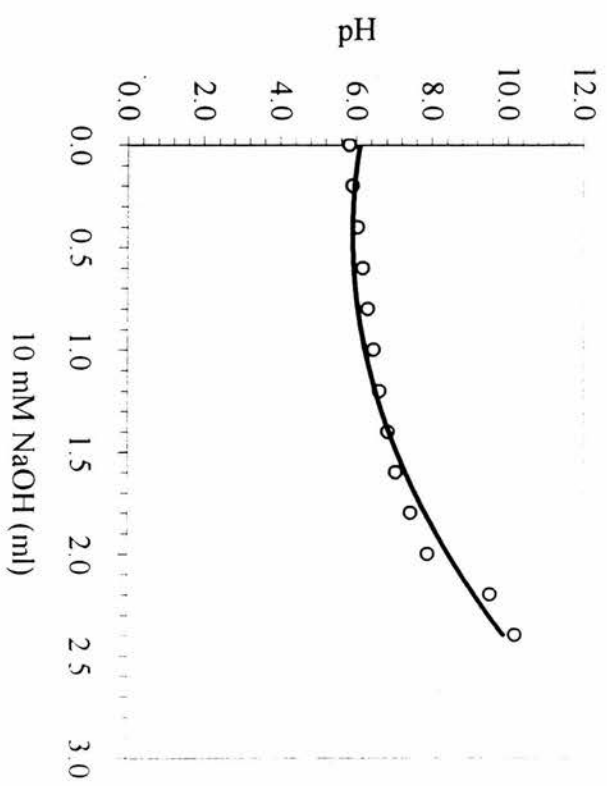
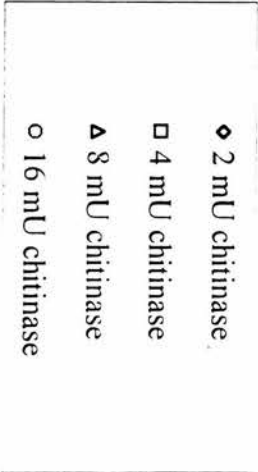
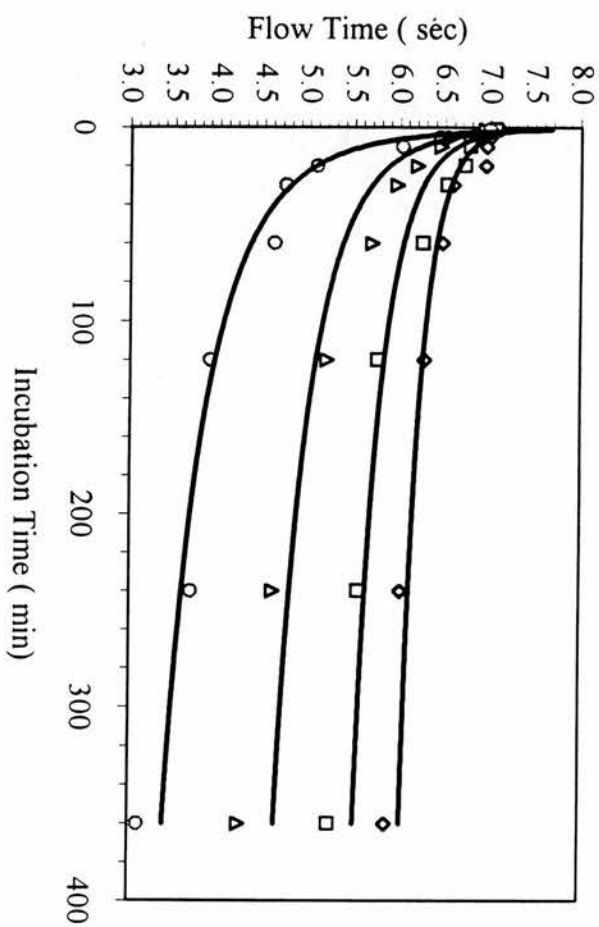


Figure 3.3 Viscosimetric assay of *Serratia* chitinase towards glycol-chitin

The reaction comprised 0.25 ml of 4% (w/v) of glycol-chitin solution, variable amounts of chitinase, 2 mU, 4 mU, 8 mU, and 16 mU, and 1 M MES buffer, pH 6.0 to complete 0.5 ml. The reaction was performed at room temperature in a microfuge tube. When the reaction was started, the flow time of the reaction was measured in a viscometer No 42 (0-0.16 ml) at different time intervals from 1 min to 360 min.



Prior to investigating the chitinase activity by colorimetric assay, it was necessary to determine the λ_{max} of the azure compound, which would be released by action of the enzyme, in order to obtain the most suitable wavelength for measurement of enzyme activity. The experiment was performed by treating the chitin-azure substrate (prepared in suspension) with concentrated sulphuric acid. In this way, the bond formed between chitin and azure molecules was broken. The azure product solubilised in the mixture obtained by centrifugation was scanned at a visible wavelength from 330-600 nm as the result shown in Figure 3.4. It showed that the azure compound absorbed over a relatively broad range of the wavelength between 518-546 nm. In this study, the wavelength of 520 nm was chosen for determination of the chitinase activity. In the colorimetric assay, the reaction was performed in duplicate. The reaction was carried out for a period of time from 1 min to 360 min. After the insoluble substrate was removed by centrifugation, the supernatant containing azure molecules was taken and measured spectrophotometrically. Results in Table 3.1 show no action of the enzyme towards the chitin-azure substrate, even if a very high amount of the enzyme up to 16 mU was added and the reaction was maintained as long as 480 min.

3.3 Expression of Chitinases in Marine Bacteria, *Vibrio*

First of all, only two species of *Vibrio*, *Vibrio alginolyticus* strain ATCC17749 and *Vibrio harveyi* strain ATCC14126, were kindly provided by the laboratory of Professor Brian Austin. They were investigated for chitinase activity. The bacteria were also studied for the effect of time course and substrates on expression of chitinases. Experiments were carried out initially by radioactive assay using [^{14}C]chitin as substrate. However, to examine the chitinases from more species of marine *Vibrio* obtained later, the enzyme activity towards [^3H]chitin was examined.

Figure 3.4 Determination of λ_{max} of azure compound

500 μl of 1% (w/v) chitin-azure solution was mixed with concentrated sulphuric acid to break the bond formed between chitin and the azure dye molecules. The supernatant was taken after vortex mixing, then centrifugation for 5 min using bench top centrifuge. The solution was measured spectrophotometrically at a visible wavelength from 330 to 600 nm.

ABSORBANCE

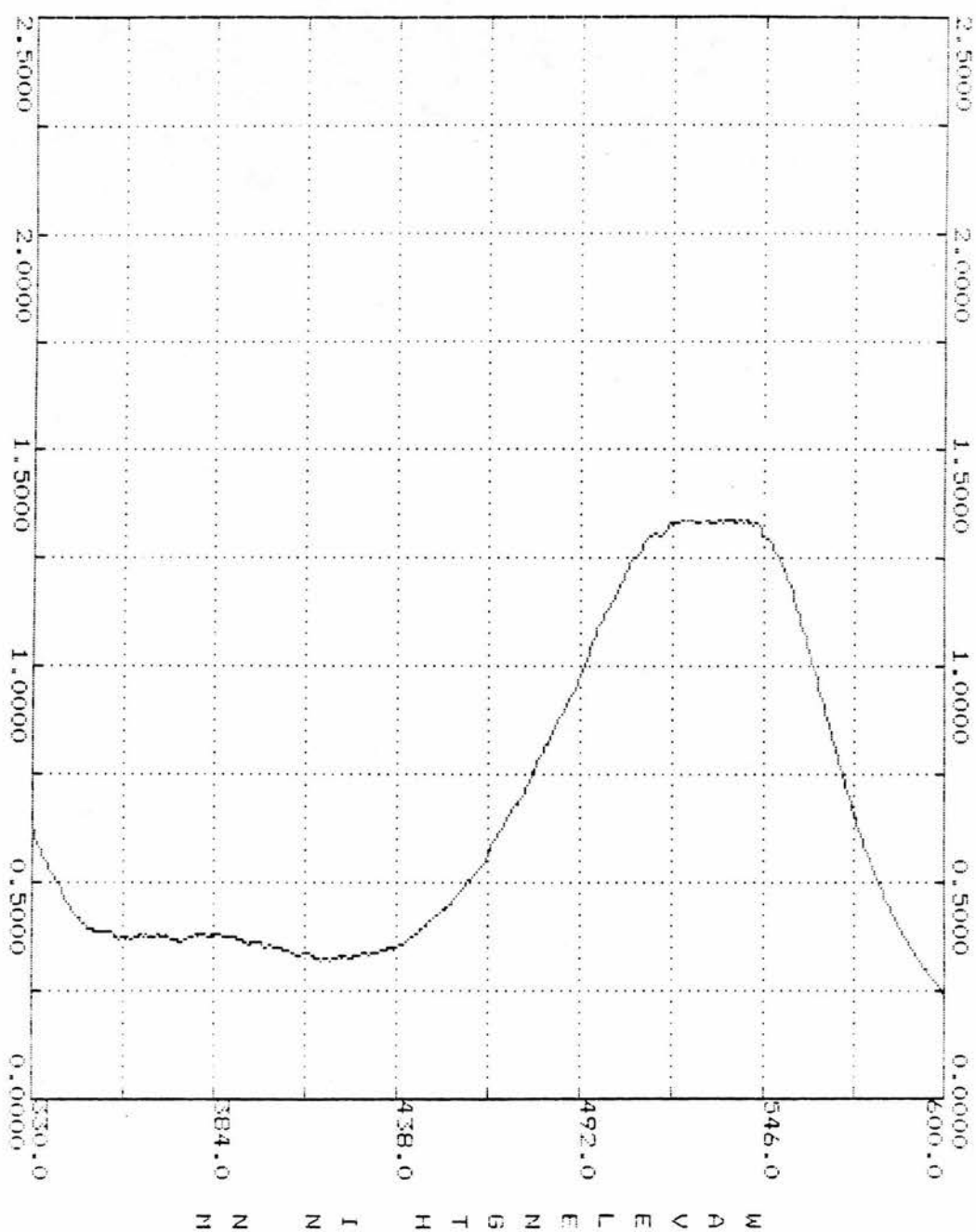


Table 3.1 Determination of *S. marcescens* chitinase by colorimetric Assay

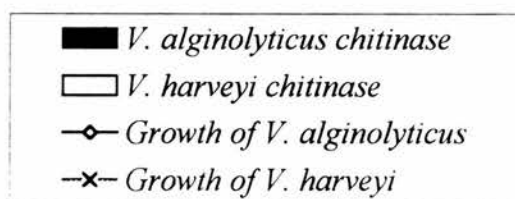
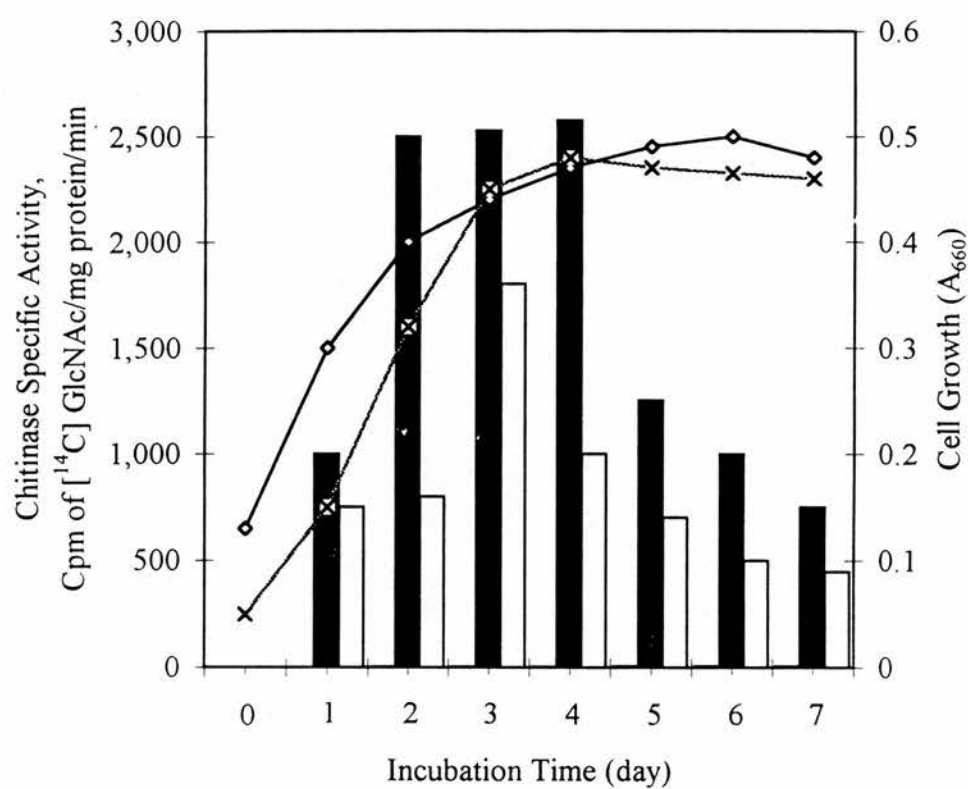
Incubation Time (min)	<u>Absorbance at 520 nm</u> Amount of chitinase used			
	2 mU	4 mU	8 mU	16 mU
0	-0.002	-0.002	-0.010	-0.010
10	-0.009	-0.008	-0.006	-0.007
20	-0.008	-0.009	-0.006	-0.009
30	-0.008	-0.005	-0.006	-0.007
60	-0.006	-0.010	-0.006	-0.007
120	-0.007	-0.007	-0.003	-0.007
240	-0.001	-0.001	-0.006	-0.005
480	-0.001	-0.005	-0.002	-0.001

3.3.1 Effect of Time Course on Chitinase Expression

Because only two *Vibrio* species, *Vibrio alginolyticus* strain ATCC17749 and *Vibrio harveyi* strain ATCC14126, were provided at initial stage, they were investigated for effect of growing times on chitinase expression. *Vibrio alginolyticus* was grown in VCM broth, pH 8.5, while *Vibrio harveyi* was grown in LB broth, pH 6.5. Single colonies of each *Vibrio* were picked into 5 ml of the appropriate medium containing 5% (w/v) chitin flakes. The cultures were incubated overnight at 30°C with 200 rpm shaking, then 0.5 ml was inoculated into 500 ml of their starting medium. Chitinase activity produced and released into the growth medium was examined every day for seven days as well as protein concentration and OD₆₆₀. The result in Figure 3.5 showed that *Vibrio alginolyticus* expressed two fold higher chitinase activity than *Vibrio harveyi*. The highest activity of *V. alginolyticus* chitinase was observed when the cells were grown in the medium for four days whereas the highest activity of *V. harveyi* chitinase was observed when the cells were grown in the medium for three days

Figure 3.5 Effect of incubation times on chitinase expression

Vibrio alginolyticus strain ATCC 17749 was grown in 500 ml VCM broth, pH 8.5. The culture medium was incubated in a 30°C shaking incubator for seven days. Samples (10 ml) were taken and centrifuged to remove cells before assaying for chitinase activity as well as protein concentration and OD₆₆₀. *V. harveyi* strain ATCC14126 was grown in LM broth, pH 6.5. The enzyme activity was examined in the same manner as for *V. alginolyticus*.



3.3.2 Effect of Chitin Substrates on Chitinase Expression

The effect of substrates on chitinase expression was studied to look for a condition for higher production of the enzyme. *Vibrio alginolyticus* was grown in VCM, pH 8.5 as described in section 2.2.2.1. The chitinase activity was measured every day for eight days. The chitinase released extracellularly was observed when the cells were grown in the medium with two different forms of chitin (swollen chitin and flake chitin) and with no chitin added. The result in Figure 3.6 showed that higher activity (about 2.5 fold) was detected when the cells were grown with 5% (w/v) of swollen chitin compared with when the cells were grown in the medium with the same amount of flake chitin. A very slight activity of the enzyme was detected when the cells were grown in the medium without any chitin added.

3.3.3 Expression of Chitinase by Fourteen Species of Marine *Vibrio*

Fourteen species of marine *Vibrio*: *V. aestuarianus*, *V. alginolyticus* 283, *V. alginolyticus* 284, *V. cambellii*, *V. carchariae*, *V. diazotrophicus*, *V. fisheri*, *V. gazogenes*, *V. harveyi*, *V. marinus*, *V. natriegens*, *V. nereis*, *V. pelagius*, and *V. splendidus*, obtained from the laboratory of Professor Brian Austin were investigated for chitinases. All the *Vibrio* species were grown in marine medium, pH 7.6 containing 5% (w/v) swollen chitin. Chitinase activity secreted by the cells into the growth medium was examined by radioactive assay towards [³H]chitin at variable growing times (1 day, 2 days, 4 days, 7 days, and 10 days) according to the method outlined in section 2.2.1.1.2. The enzyme was determined when the cells were grown in the medium with and without swollen chitin as shown in Figure 3.7. The values of the enzyme activity detected at day1, day4, and day7 when the cells of the twelve *Vibrios* (except *V. marinus* and *V. splendidus*) were grown in medium with and without chitin are displayed in Table 3.2. In summary, it was found that most the *Vibrio* species

Figure 3.6 Effect of chitin substrates on chitinase expression

Vibrio alginolyticus strain ATCC17749 was grown in VCM broth, pH 8.5 containing 5% (w/v) two different forms of chitin, chitin flakes and swollen chitin. Samples of the cell culture (10 ml) were taken every day for eight days, then centrifuged for 5,000 rpm for 20 min at 4°C to remove the cell pellet. The supernatant was assayed for chitinase activity as well as protein concentration. The enzyme activity was also determined when the cells were grown in the medium without chitin added.

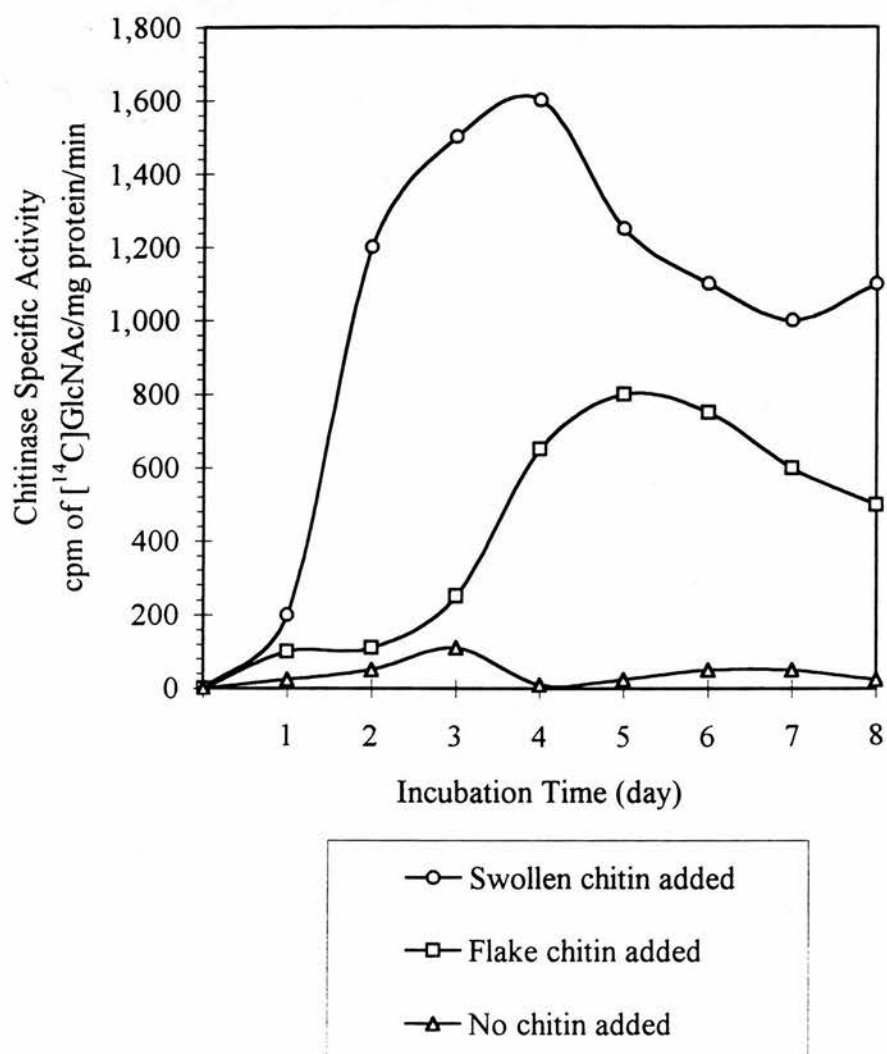


Figure 3.7 Expression of chitinases by fourteen species of marine *Vibrio*

Fourteen species of marine *Vibrio*; *V. aestuarianus*, *V. alginolyticus* 283, *V. alginolyticus* 284, *V. cambellii*, *V. carchariae*, *V. diazotrophicus*, *V. fisheri*, *V. gazogenes*, *V. harveyi*, *V. marinus*, *V. natriegens*, *V. nereis*, *V. pelagius*, and *V. splendidus*, were grown according to the procedure described in materials and methods (section 2.2.2.1). The cell culture of each *Vibrio* species (10 ml) was taken at time intervals of 1 days, 2 days, 4 days, 7 days, and 10 days, and centrifuged to remove the cell pellet. The supernatant was examined for chitinase activity towards [³H]chitin as well as protein concentration. Expression of chitinases when the cells were grown in the medium with 5% (w/v) swollen chitin is shown in Figure 7A whereas expression of chitinases when the cells were grown in the medium without chitin added is shown in Figure 7B.

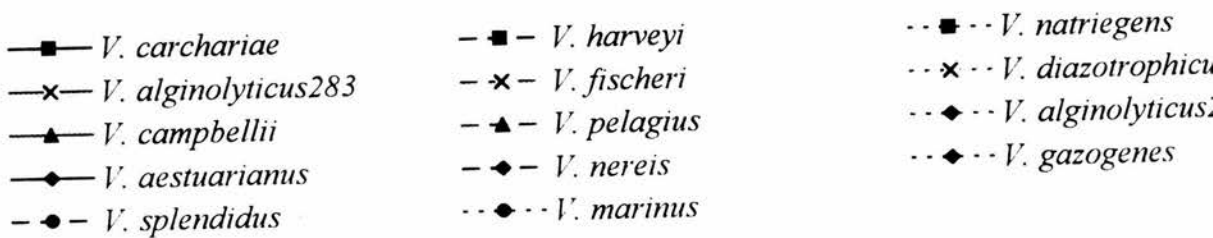


Table 3.2 Chitinase activity obtained from twelve species of *Vibrio*

<i>Vibrio</i> sp.	Growing Time (day)					
	Day1		Day4		Day7	
	+SC*	-SC	+SC	-SC	+SC	-SC
<i>V. carchariae</i>	0.55 **	0.24	0.51	0.33	0.51	0.32
<i>V. harveyi</i>	0.05	0.27	0.26	0.13	0.29	0.14
<i>V. natriegens</i>	0.02	0.04	0.12	0.18	0.08	0.07
<i>V. alginolyticus</i> 283	0.02	0.09	0.78	0.16	0.38	0.06
<i>V. fischeri</i>	0.04	0.09	0.22	0.12	0.10	0.16
<i>V. diazotrophicus</i>	0.04	0.02	0.01	0.12	0.06	0.08
<i>V. campbellii</i>	0.02	0.04	0.43	0.14	0.41	0.14
<i>V. pelagius</i>	0.01	0.13	0.11	0.01	0.06	0.09
<i>V. alginolyticus</i> 284	0	0.01	0.13	0.10	0.31	0.06
<i>V. aestuarianus</i>	0.15	0.20	0.11	0.25	0.24	0.19
<i>V. nereis</i>	0.04	0.07	0.10	0.07	0.19	0.20
<i>V. gazogenes</i>	0.01	0.05	0.05	0.08	0.09	0.16

SC represents 5% (w/v) swollen chitin added into the growth medium; ** The enzyme activity is given in U/mg protein, The highest enzyme activity detected in each growing time is given in open boxes.

expressed higher chitinase activity when the cells were grown in the medium with swollen chitin added. Moreover, among the fourteen species studied, only three species, *V. alginolyticus* 283, *V. cambellii*, and *V. carchariae*, expressed high chitinase levels whereas only slight activity was detected in the other twelve species. *V. alginolyticus* produced the highest activity (0.78 U/mg protein) at four days of incubation followed by *V. carchariae*, which produced slightly lower activity (0.55 U/mg protein) at one day of incubation. *V. cambellii* produced moderate activity (0.43 U/mg) at four days of incubation. It was noticed that much lower activity was detected from *V. harveyi*.

3.3.4 Expression of Chitinases on Chitin Plates

To investigate the capability of chitinases secreted outside the cells to degrade chitin on a solid medium, twelve species of marine *Vibrio*, except *V. marinus* and *V. splendidus*, were grown on chitin agar plates. Expression of chitinases was indicated by the formation of clear zones around the stab sites. It was possible to detect degradation of chitin on agar plates after three days of incubation. In addition, the results in Figure 3.8A, 3.8B, and 3.8C showed that *V. alginolyticus* 283 produced the highest activity on chitin plate, followed by *V. alginolyticus* 284, *V. fisheri*, and *V. carchariae*, respectively. *V. harveyi*, *V. cambellii*, *V. neries*, and *V. pelagius* produced slight chitinase activity on chitin plate. No chitin degradation was observed in colonies of *V. diazotrophicus*, *V. gazogenes*, *V. aestuarianus*, and *V. natriegens*, even though the colonies were incubated and stored at 4°C for a month.

3.4 Conclusions and Discussion

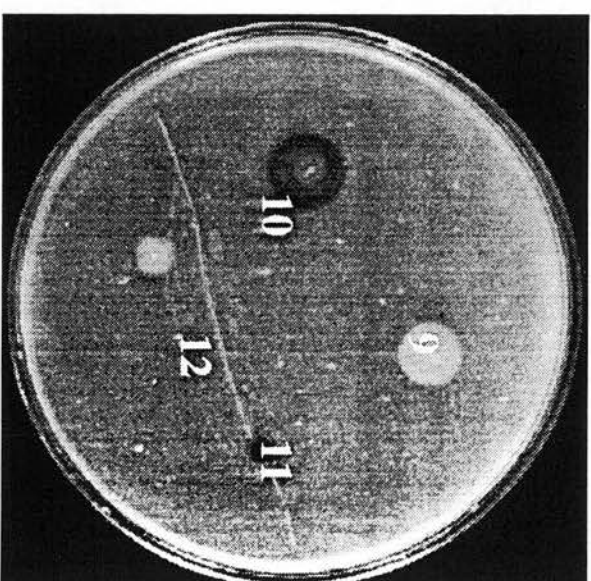
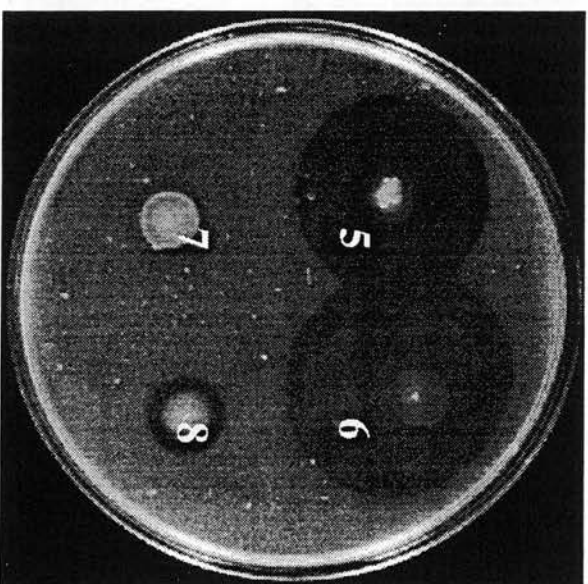
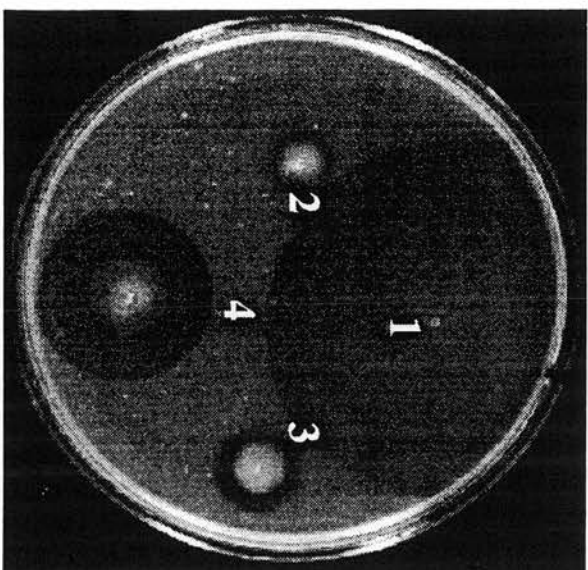
3.4.1 Development of an Assay Procedure for Chitinase Studies

Comparing three different assay methods, radioactive assay using [¹⁴C]chitin, viscometric assay, and colorimetric assay, the first method showed higher sensitivity

Figure 3.8 Expression of chitinases on chitin agar plate

Single colonies of marine *Vibrio*, except *V. marinus* and *V. splendidus*, were inoculated into suitable media (5 ml samples), and grown up as the conditions mentioned in section 2.2.2.5. The cell culture was stabbed on to marine agar plate containing 1% (w/v) swollen chitin. The cells were incubated three days at 30°C, then stored at 4°C for a month. The clear zone around the stab sites was observed. *Vibrio gazogenus* was a dark colony, and did not produce a clear zone.

Well	1	<i>V. alginolyticus</i> 283	Well	7	<i>V. natrieigen</i>
	2	<i>V. harveyi</i>		8	<i>V. nereis</i>
	3	<i>V. cambellii</i>		9	<i>V. diazotrophicus</i>
	4	<i>V. carchariae</i>		10	<i>V. pelagius</i>
	5	<i>V. fisheri</i>		11	<i>V. gazogenes</i>
	6	<i>V. alginolyticus</i> 284		12	<i>V. aestuarianus</i>



in determination of commercial chitinase from *Serratia marcescens*. It was possible to detect as little as 1 mU of enzyme. The viscometric method showed high sensitivity in determination of chitinase but still not as sensitive as when compared with the radioactive assay. Moreover, this method showed limitations in terms of a large-scale determination. It is rather troublesome and time consuming to determine the chitinase activity of numerous samples.

The colorimetric assay using chitin-azure substrate was unsuitable to investigate chitinase activity as no action of the enzyme towards the substrate was detected, even though the reaction was carried out as long as 360 min, and the amount of the enzyme used was as high as 16 mU. The results obtained were different from that reported previously (Hackman and Goldberg, 1964). The degradation capability of a plant chitinase was illustrated on either Red-G dyed chitin or Remazol Brilliant violet 5R dyed chitin. Chitinase activity has also been determined by colorimetric assay by measuring reducing sugars released from colloidal chitin by the method modified from Schales (Imoto and Yagishita, 1971) but the colloidal chitin in the suspension is heterogeneous, and consequently the method lacks accuracy and reproducibility.

Even though the radioactive assay showed limitation in terms of cost of substrate preparation, the method was reliable and very sensitive, and convenient compared with other methods examined.

In conclusion, among the three different assay methodologies evaluated, the radioactive assay appeared to be the most suitable procedure in terms of its sensitivity, convenience and efficiency. The assay was therefore chosen for chitinase study in this project.

3.4.2 Expression of Chitinase from Marine bacteria, *Vibrio*

An initial study of the effect of substrates showed that the expression of *V. alginolyticus* chitinases required a chitin substrate as an inducer. Only very slight

activity was detected when the cells were grown in the medium without chitin, while a strikingly higher level of the enzyme was detected when the cells were grown in the medium with chitin added. The result obtained was strongly supported by previous reports (Monreal and Reese, 1968; Huang *et al.*, 1996; Woo *et al.*, 1996; and Svitil *et al.*, 1997).

The form of chitin substrate affected the expression of chitinase. Higher activity was detected when the cells were grown in the medium with swollen chitin added compared with when flake chitin was added. This might be explained that the cells degraded the acid-treated chitin more effectively, then took up and utilised by the cells more rapidly than they did with untreated chitin.

The results clearly showed that *V. alginolyticus* or *V. harveyi* expressed the enzyme, as an increase of chitinase activity with growing time (up to 4 days for *V. alginolyticus* and 3 days for *V. harveyi*) (see Figure 3.5) was detected. The enzyme activity gradually decreased when the cells were continued growing, while the cell growth remained linear. The results were in good agreement with the other bacterial species determined later. The decline of the enzyme activity after it reached the maximum level might be a result of catabolite repression. Even though chitin in the growth medium induced chitinase expression, its degraded products also inhibited the chitinase activity (Soto-Gil and Zyskind, 1984). Decrease in chitinase activity was suggested as a result of proteolytic enzyme activity released from the cells (Woo *et al.*, 1996). Therefore, to maximise chitinase production, time for bacterial culture appears to be important and needs to be optimised in order to balance between the effects of chitin induction and inhibition from chitin degraded products.

It should also be addressed here that the chitinase activity detected in this study was a combination effect of chitinase expression from the multiple gene system as suggested in previous studies (Fuchs *et al.*, 1986; Jones *et al.*, 1986; Harpster and Dunsmuir, 1989; Watanabe *et al.*, 1990; Mayashita *et al.*, 1991; and Stivil *et al.*, 1997), and adsorption to chitin added into the growth medium. In latter case, the value of the

enzyme activity obtained when the cells were grown in the medium with chitin added was likely to be less than the genuine value. Effects of chitin on the enzyme molecules were clearly observed when chitin affinity chromatography was used in purification of *V. carchariae* chitinase as discussed in chapter 4 (sections 4.6 and 4.7.5).

However, with regard to chitinase expression determined by radioactive assay and chitin plate assay, *V. alginolyticus* 283 appeared to be the most suitable species for further studies of chitinase as it showed the highest activity on both assays. *V. carchariae* could be an alternative source for chitinase as well.

Chapter 4

Purification and Characterisation of *V. carchariae* Chitinase

4.1 Introduction

Current investigations of microbial chitinases have been directed towards the selection of microorganisms and culture conditions leading to high yields of chitinases, and to the purification of these enzymes for further characterisation and/or studies at the DNA level.

In bacteria, a number of studies have reported the purification and characterisation of chitinases from various sources. It is typical that each bacterium secretes more than one type of chitinase. *Bacillus circulans* WL-12 secretes at least six major chitinases, which differ in enzyme properties (Watanabe *et al.*, 1997). Five unique chitinolytic enzymes have been isolated from *Serratia marcescens*, although not all of their genes have been cloned (Jones *et al.*, 1986; Fuchs *et al.*, 1986; Harpster and Dunsmuir, 1989). Three separate chitinase genes have been identified in *Streptomyces lividans* (Miyashita *et al.*, 1991). These studies reveal that chitinases with diverse enzymic properties may be encoded by separate genes, or may result from proteolytic processing. It is proposed that a bacterium produces different chitinases to enable efficient hydrolysis of the different forms of chitin found in nature. Previous reports have suggested that a single chitinase is not equally efficient hydrolysing α and β forms of chitin. For example, Shigemasa and colleagues (Shigemasa *et al.*, 1994) found that a chitinase isolated from *Bacillus sp.* strain P1-7S degraded β -chitin more efficiently than α -chitin. Svityl and colleagues (Svityl *et al.*, 1997) showed that *Vibrio harveyi* secreted several chitin-degrading proteins into the cell culture. This bacterium was found to have higher growth rate and more chitinase activity when grown on β -

chitin (isolated from squid pen) than on α -chitin (isolated from snow crabs). Therefore, to investigate a particular chitinase of interest from a bacterial source, it seems to be essential to isolate the enzyme away from other contaminating chitinases.

It is apparent from various studies that chitinases from different sources required different methods to obtain the purified protein. Only two steps, ammonium sulphate precipitation and ion-exchange chromatography on DEAE-Sephadex were used in purification of a chitinase from *Serratia marcescens* (Monreal and Reese, 1968). However, an additional step of gel filtration chromatography on Sephadex-G100 was required to obtain a purified chitinase from a marine luminous bacterium, *V. fisheri* strain COT-A136 (Fukasawa *et al.*, 1992). To isolate ChiA from *Aeromonas hydrophila* H2330 (Hiraga *et al.*, 1997), four steps including ammonium sulphate precipitation, followed by DEAE-Sepharose, Sephadex G-100, and isoelectric focusing, were used. In the marine bacterium, *Alteromonas sp.* strain O-7, three steps including first ion-exchange chromatography on DEAE-Toyopearl, followed by Sephadex G-100, then a second ion-exchange chromatography on DEAE-Toyopearl, were used to isolate ChiA (Tsujiibo *et al.*, 1992). However, there have been no reports of using chitin affinity chromatography in the purification of bacterial chitinases, even though chitin affinity has appeared to be a useful step in purification of chitinases from plant sources (Boller *et al.*, 1983; Kragh *et al.*, 1991; and Yamamoto *et al.*, 1995).

In this chapter, an attempt to isolate a chitinase from the marine bacterium, *V. alginolyticus* is described. The successful purification of ChiA from *V. carchariae* is also detailed.

4.2 Attempts to Develop a Purification Scheme for *Vibrio alginolyticus* Chitinase

Expression of chitinases by two marine *Vibrios* (*V. alginolyticus* strain ATCC17749 and *V. harveyi* strain ATCC14126) into the broth medium clearly showed that *V. alginolyticus* secreted higher chitinase activity than *V. harveyi*. Therefore, *V. alginolyticus* was judged to be a more suitable source for further studies of the

enzyme. Purification for the *V. alginolyticus* chitinase was tried with three steps including ammonium sulphate precipitation, followed by ion-exchange chromatography on DEAE-Sephadex A-50, and chitin affinity chromatography.

4.2.1 Ammonium Sulphate Precipitation

Ammonium sulphate precipitation was used in the first purification step. To determine the appropriate concentration range for quantitative precipitation of chitinase, the growth medium of *V. alginolyticus* was fractionated into two ranges of ammonium sulphate saturation; 0-35% saturation, and 35-70% saturation. Fractions (the precipitate and the supernatant obtained from 35-70% ammonium sulphate saturation) were tested for chitinase activity as well as protein concentration, as summarised in Table 4.1. The total chitinase activity was very well recovered, whereas more than half the protein content was removed when the growth medium was fractionated at 35-70% ammonium sulphate saturation. The yield obtained from this step was 94%. The specific activity was determined as 0.55 U/mg, giving two-fold purification for this step.

4.2.2 Chromatography on DEAE-Sephadex A-50

Ion-exchange chromatography on DEAE-Sephadex A-50 was used as the following purification step. Chromatography was performed as described in materials and methods (section 2.2.3.2). After 35-70% ammonium sulphate precipitate fraction was dialysed extensively against 10 mM potassium phosphate buffer, pH 7.0 to remove ammonium sulphate, and the ionic strength was checked until it was low enough. The sample was applied to the column, then washed thoroughly with 10 mM potassium phosphate buffer, pH 7.0, followed by a gradient of 0-0.5 M sodium chloride. Profiles of chitinase activity, protein (A_{280}), and ionic strength are shown in Figure 4.1 and the purification result is summarised in Table 4.1.

Figure 4.1 Ion-exchange chromatography on DEAE-Sephadex A-50 of the 35-70% ammonium sulphate precipitate fraction

Dialysed sample obtained from the ammonium sulphate precipitation step (5 ml) containing 43 mg protein was applied to DEAE- Sephadex A-50 column (2 x 8 cm) pre-equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The column was washed with 3 column volumes of the same buffer before a gradient of 0-0.5 M sodium chloride prepared in the same buffer (eight column volumes) was applied. Fractions (2 ml) were collected and assayed for chitinase activity as well as for protein content (A_{280}). The ionic strength of every fifth fraction was determined.

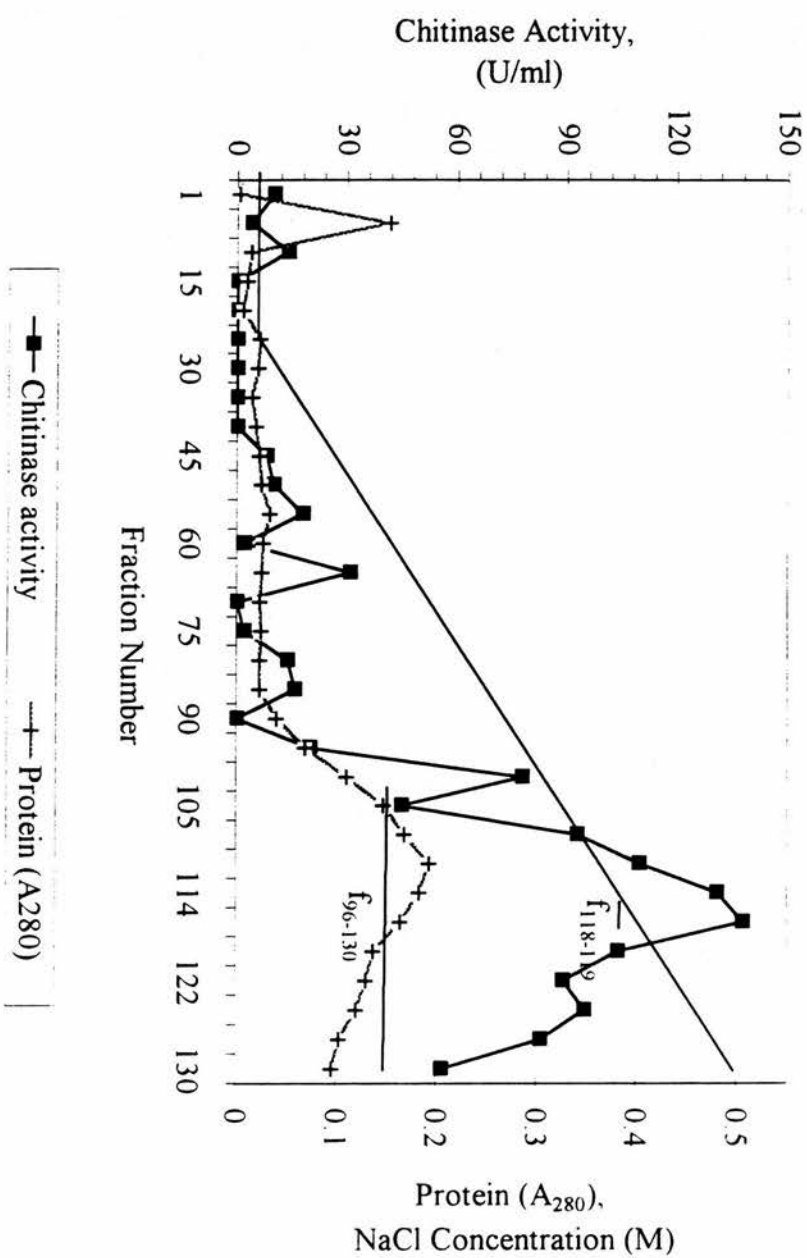


Table 4.1 Partial purification of chitinase from *V. alginolyticus* strain ATCC17749

Step	Total Protein (mg)	Total Activity (Units)	Specific Activity (U mg ⁻¹)	Fold Purification	% Recovery
Growth Medium	90	25.3	0.28	-	100
35-70% Ammonium ppt.	43	24.8	0.58	2.1	94
35-70% Ammonium sup.	49	0	0	0	0
DEAE-Sephadex A-50					
Pooled f ₁₈₋₁₁₉	0.6	0.62	1.0	3.6	2.4
Pooled f ₉₆₋₁₃₀	25	20.4	0.82	2.9	80
Total pool	25.6	21.0	0.82	2.9	82

From the profiles, it was shown that most proteins were bound to the column and were eluted with a sodium chloride gradient. Chitinases were found to bind quite tightly to the column, as a relatively high concentration of sodium chloride (about 0.4 M) was required to elute the enzymes from the column.

Based on the protein and the chitinase profiles, the column appeared to remove some proteins that were not bound or bound weakly, while the chitinase was still retained in the column. Result in Table 4.1 showed that the pool (f₁₁₈₋₁₁₉) containing high chitinase activity provided slightly higher specific activity (1.0 U/mg) than the pool (f₉₆₋₁₃₀), which gave specific activity of 0.82 U/mg. The total yield obtained from the two pools was 82% with 2.9 fold purification. This step removed 40% of proteins from the previous step.

4.2.3 Affinity Chromatography on Chitin

Even though ammonium sulphate precipitation and ion-exchange chromatography dramatically increased the purification of chitinase, SDS-PAGE showed that a further step was still required to obtain purified chitinase. After sample obtained from ion exchange chromatography step was applied to chitin affinity column, chromatography was performed as described in section 2.2.3.3. The result (data not shown) showed that chitinase bound so tightly to chitin that it could not be eluted by 0.1 M *N*-acetylglucosamine, or 5% (v/v) acetic acid, but much stronger conditions such as 20% (v/v) acetic acid, or 6 M guanidine HCl were required. However, as a consequence of using such strong conditions, no enzyme activity was detected after elution.

4.3 Purification of *V. carchariae* Chitinase

Since purification of *V. alginolyticus* proved to be difficult, *V. carchariae*, which also showed a high level of chitinases, was considered to be an alternative source for enzyme isolation. As it released fewer proteins into the growth medium (as judged by

SDS-PAGE (data not shown), so it was assumed that a purification scheme for *V. carchariae* chitinase might be easier to achieve.

4.3.1 Effect of Amount of Chitin on Chitinase Expression from *V. carchariae*

The effects of different concentrations of swollen chitin on the expression of chitinase from *V. carchariae* were studied in order to obtain higher yields for large-scale preparation of the enzyme. The growth medium was tested for chitinase activity as well as protein concentration after the cells were grown 48 h at 30°C in growth media containing variable concentrations of swollen chitin from 0.5% (w/v) to 10% (w/v). The result in Figure 4.2 showed that the highest level of chitinase was observed when the cells were grown in the medium containing 2.5% (w/v) chitin. A decrease in the enzyme activity was observed when concentrations of chitin were higher than 2.5% (w/v). The enzyme activities (82% and 7%) were found when concentration of chitin added was 5% (w/v) and 10% (w/v), respectively compared with when 2.5% (w/v) chitin was added. This suggested that high amounts of chitin affected measurement of the enzyme activity.

4.3.2 Effect of Concentrations of Guanidine HCl on Release of Chitinase from Chitin Affinity Chromatography

Different concentrations of guanidine HCl were tested in order to optimise the yield in the chitin affinity step. The crude enzyme bound to chitin was incubated with different concentrations of guanidine HCl from 0.1 M to 6 M at certain period of time. After elution, the guanidine-eluted protein solution was dialysed extensively overnight against 20 mM potassium phosphate, pH 7.0 at 4°C, and the enzyme activity was determined as well as protein concentration. The result in Figure 4.3 showed that increasing chitinase activity was released with concentrations of guanidine HCl up to 2 M. However, the activity began to decrease when concentrations of guanidine HCl tested were higher than 2 M. The highest relative

Figure 4.2 Effect of amount of chitin on chitinase expression from *V. carchariae*

V. carchariae was cultured 48h at 30°C with 200 rpm shaking in the media containing variable amounts of swollen chitin, and the chitinase activity and protein content were measured.

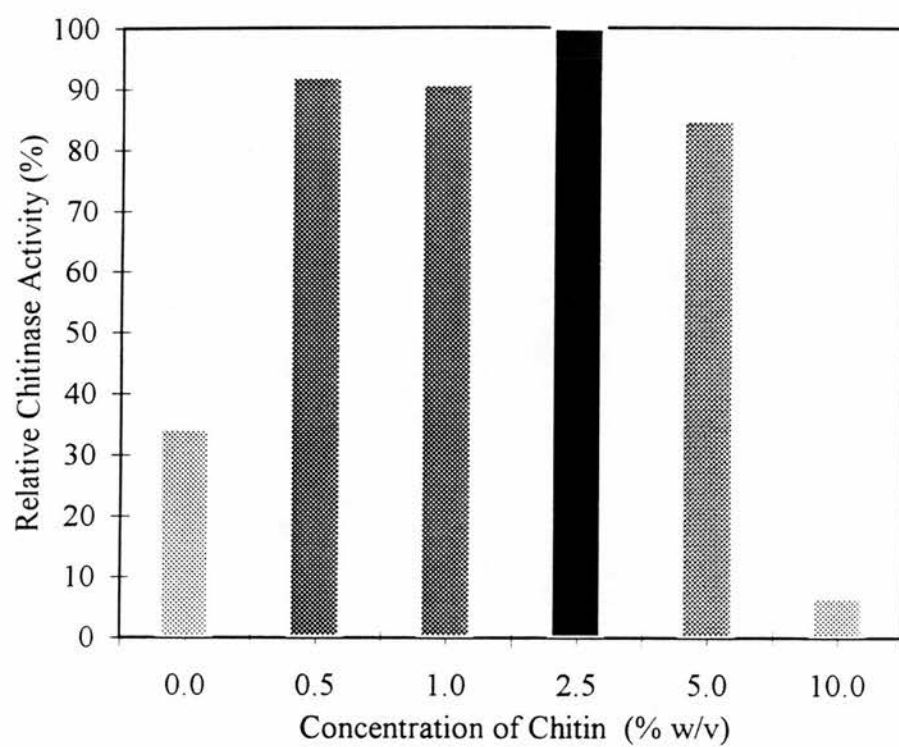
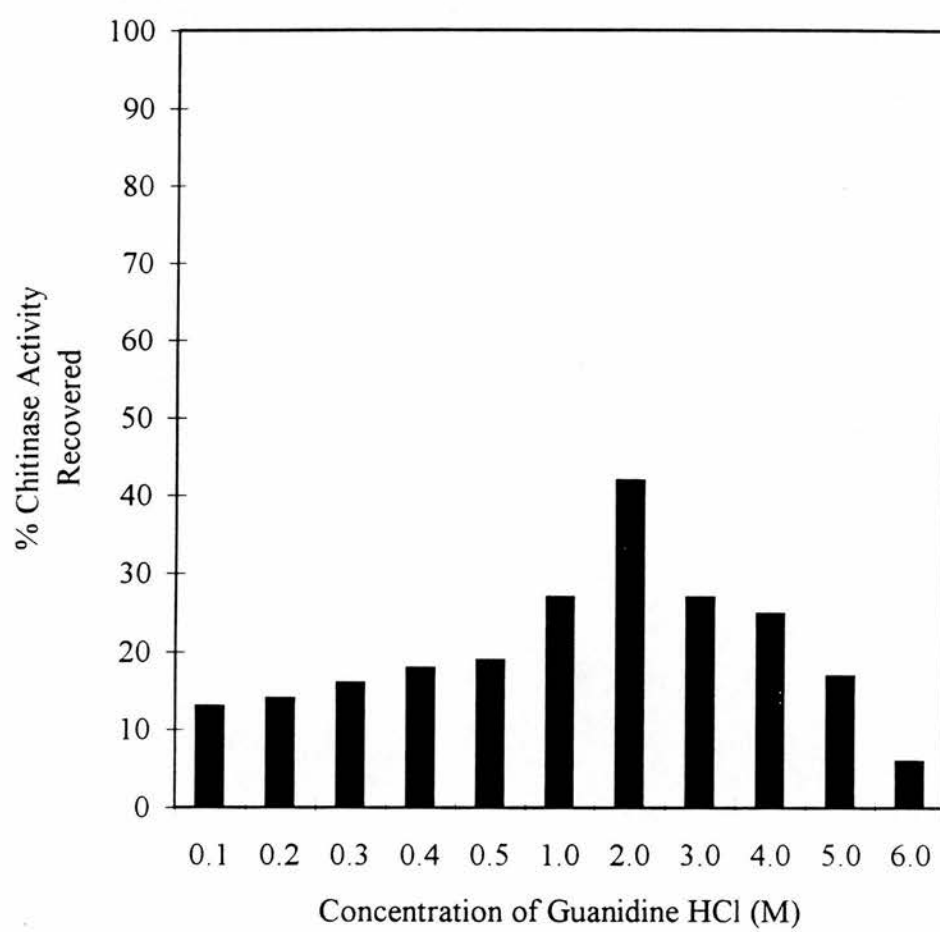


Figure 4.3 Effect of concentrations of guanidine HCl on release of chitinase from chitin affinity

One ml samples (the growth medium of *V. carchariae*) were added into microfuge tubes containing 0.1 g swollen chitin, then mixed by vortexing rapidly for 1 min. After centrifugation, the medium was discarded and variable concentrations of guanidine HCl (0.1-6.0 M) were added into separate tubes. After vortex mixing for 1 min, the supernatant was collected by centrifugation. The chitinase activity of each supernatant was measured.



activity (42%) remained at 2 M guanidine HCl (see section 4.7.2 for detailed discussion).

4.3.3 A Complete Purification of *V. carchariae* Chitinase

A complete purification scheme of *V. carchariae* chitinase developed in this study comprised three steps as described in section 2.2.4.3. The steps included batch-wise chitin affinity chromatography, followed by Sephacryl S200 HR column, then Mono Q FPLC. The purification results are summarised in Table 4.2. In summary, the chitin affinity step removed a huge amount of the proteins (90% of total proteins expressed into the growth medium), while 26% of chitinase activity remained after elution with 2 M guanidine HCl. The specific activity obtained from this step was 1.45 U mg⁻¹, giving a 2.3-fold purification. SDS-PAGE (see Figure 4.7) showed that a protein bound that migrated approximately at the same position as BSA (M_r 66,000) was the major component.

The protein solution obtained after the chitin affinity step, designated "CA" fraction", was dialysed extensively against 20 mM potassium phosphate buffer, pH 7.0 to remove guanidine HCl, then concentrated to a small volume by precipitating with ammonium sulphate. The concentrated CA fraction was applied to a Sephacryl S200 HR column. Profiles of chitinase activity and A₂₈₀ are shown in Figure 4.4A. From the profiles, it was found that the column removed higher molecular weight proteins from the protein sample. Results summarised in Table 4.2 showed that approximately 80% of total proteins were removed, with 8% of chitinase activity remaining and 3.2 fold purification at this stage. The native molecular weight of the chitinase was also determined using the same column. Compared with the three molecular weight markers; phosphorylase b, bovine serum albumin (BSA), ovalbumin, and D-glyceraldehyde-3-phosphate dehydrogenase, calibrated previously, the native molecular weight of chitinase was estimated to be 66,000 (Figure 4.4B). SDS-PAGE (see Figure 4.7) showed that a number of minor protein bands were still found in the sample and a further purification step was required.

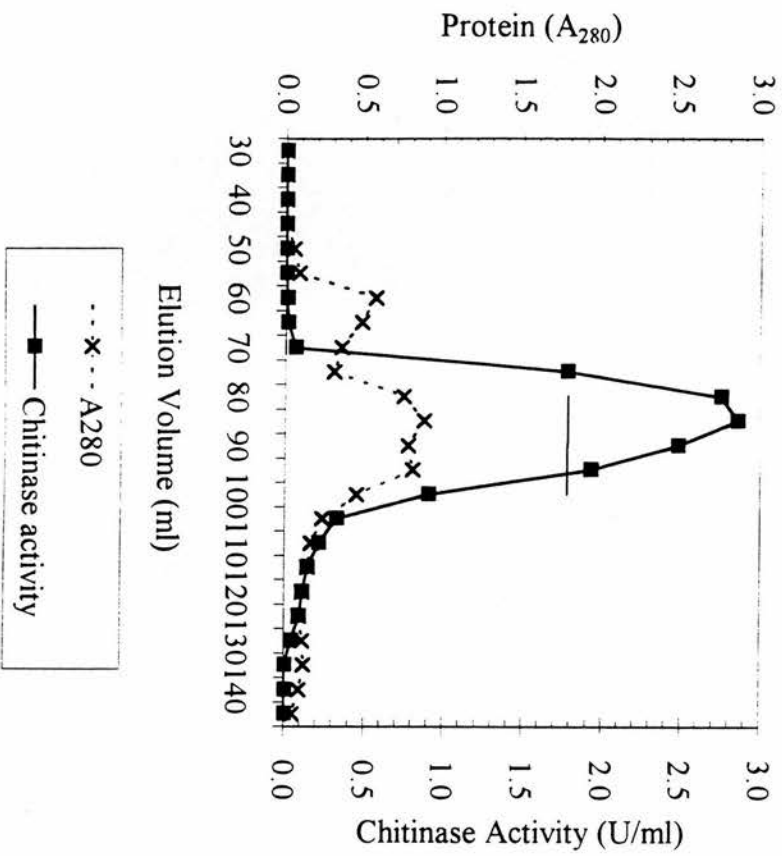
Table 4.2 A Complete purification of *V. carchariae* chitinase

Step	Total Protein (mg)	Total Activity (Units)	Specific Activity (U mg ⁻¹)	Fold Purification	% Recovery
Growth Medium (GM)	670	420	0.63	-	100
Chitin Affinity (CA)	76	110	1.45	2.3	26
Sephacryl S200 (S200)	12	34	2.8	3.2	8
Mono Q fplc (Mono Q)	3.9	19	4.9	7.8	4.5

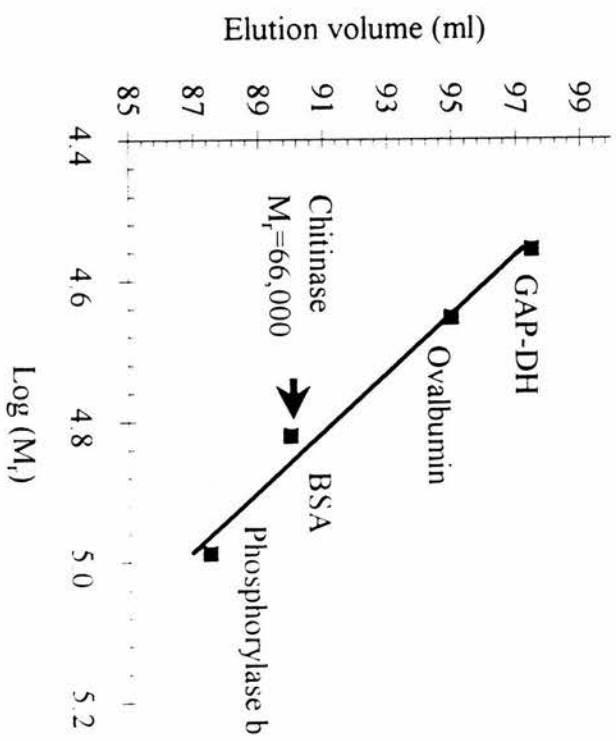
Figure 4.4 Gel filtration on Sephacryl S200 HR

A two ml sample of concentrated CA fraction (76 mg) was applied onto Sephacryl S200 HR column (2 cm x 75 cm) pre-equilibrated in 20 mM potassium phosphate buffer, pH 7.0 containing 50 mM NaCl. The column was eluted with the same buffer with a constant flow rate of 10 ml/h. Fractions (2.5 ml) were collected and assayed for chitinase activity as well as protein content (A_{280}). Profiles of chitinase activity are shown in Figure 4.4A. Determination of native molecular weight is shown in Figure 4.4B.

A)



B)



The final purification step was fplc. A Superose 12 column was initially used but it was found apparently that the enzyme interacted with the packing material, and as a result, retardation of the chitinase was encountered. Rather broad protein and activity peaks were also obtained. Judged by SDS-PAGE (Figure 4.5), and assay results (data not shown), the step was unlikely to improve the purification.

Anion-exchanger (Mono Q) FPLC was tested. The protein profile of Mono Q FPLC is shown in Figure 4.6A. Fractions of the protein peak were collected and assayed for chitinase activity. The chitinase-containing fractions were eluted by a gradient of 0.4-0.45 M sodium chloride. The step removed all contaminating proteins, as shown by a single protein band at molecular weight of 63,000 on SDS-PAGE (Figure 4.6B). Specific activity (5 U mg^{-1}) was obtained, with an over all yield of 4.5% and 7.8 fold-purification. After the final step, a highly purified chitinase showed 92% purity as estimated from a gel densitometer (data not shown). Total protein obtained from the initial 1.9 litres culture was approximately 4.0 mg.

Protein patterns obtained from each purification step were analysed by SDS-PAGE as revealed in Figure 4.7A. The subunit molecular weight of chitinase was determined under denaturing conditions based on the standard proteins mentioned in section 2.2.6. The M_r value of chitinase was calculated to be 63,000 (Figure 4.7B).

The purified chitinase obtained after the Mono Q FPLC step and shown a single protein band on SDS-PAGE was tested for chitinase activity towards the fluorescent substrates (4-MU-[GlcNac]₂), and (4-MU-[GlcNac]₄) under non-denaturing electrophoresis conditions (non-denaturing PAGE). Three protein bands (Figure 4.8A, and 4.8B) were observed and all three bands possessed chitinase activity towards the two substrates (see section 4.7.3 for discussion).

Figure 4.5 SDS-PAGE of Superose FPLC fractions

Fractions (15 μ l) obtained from Superose fplc was applied onto a SDS-PAGE gel. Electrophoresis was performed according to materials and methods (section 2.2.6)

Lane	Sample
1	low molecular weight standard markers
2	pooled chitinase fraction from Sephacryl S200 HR column
3	Superose fraction no. 7
4	Superose fraction no. 8
5	Superose fraction no. 9
6	Superose fraction no. 10

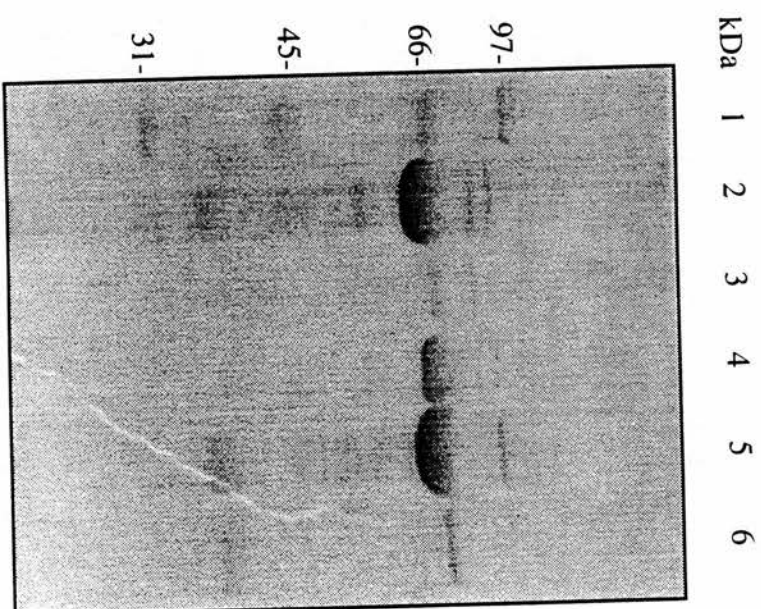
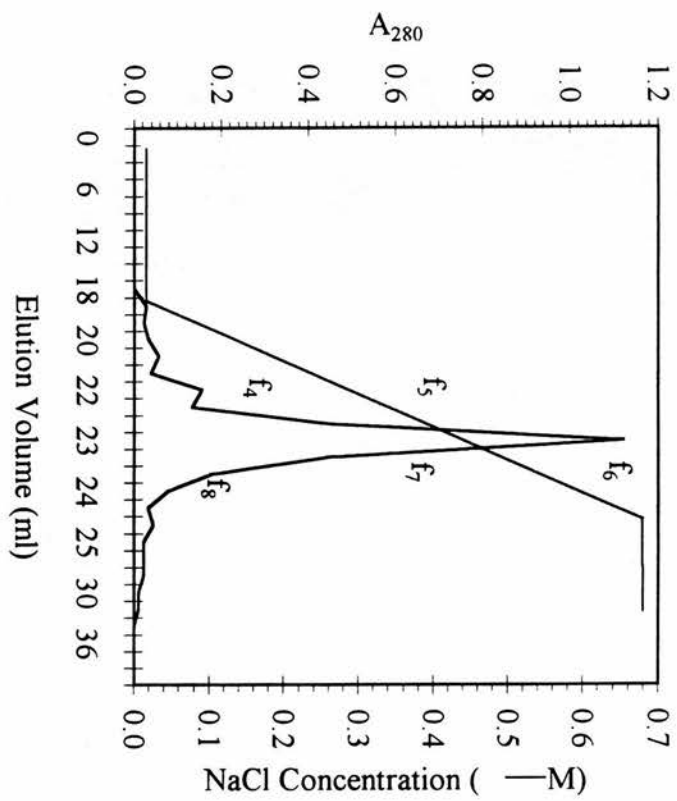


Figure 4.6 Anion-exchange chromatography on Mono Q FPLC

The f_{34-40} pool (18.2 ml) containing 8 mg protein obtained from the Sephacryl S200 HR column was dialysed extensively against 20 mM potassium phosphate buffer, pH 7.0. After centrifugation to remove undissolved material, the sample (450 μ l) was injected onto a Mono Q column using a Pharmacia FPLC [®] system. The column was pre-equilibrated in 20 mM potassium phosphate buffer, pH 7.0 and it was accomplished in 8 repeated cycles of 200 μ l sample each. The column was washed with 10 ml of the equilibrating buffer before a gradient of 0-0.7 M sodium chloride was applied. A flow rate of 1.0 ml/min was maintained with a constant pressure of 1.5 mPa. A chart speed of 0.5 ml/min was used. Fractions (0.5ml), where the protein profile was observed, were collected and assayed for chitinase activity as well as protein content (A_{280}) (Figure 4.6A). Fractions (15 μ l) were analysed by SDS-PAGE (Figure 4.6B).

A)



B)

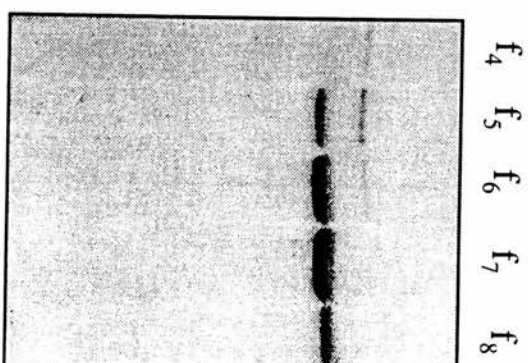
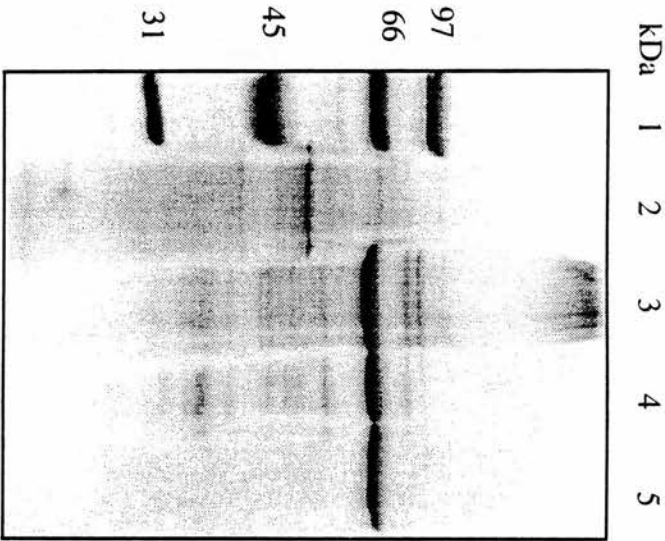


Figure 4.7 SDS-PAGE characterisation of chitinase purification fractions

Samples obtained from each purification step were analysed by SDS-PAGE (Figure 4.7A). The subunit molecular weight of *V. carchariae* chitinase was also determined (Figure 4.7B).

Lane	Sample
1	low molecular weight protein standard
2	concentrated growth medium (GM)
3	Chitin affinity fraction (CA)
4	Sephacryl S200 HR filtration fraction (S200)
5	Mono Q FPLC fraction (Mono Q)

A)



B)

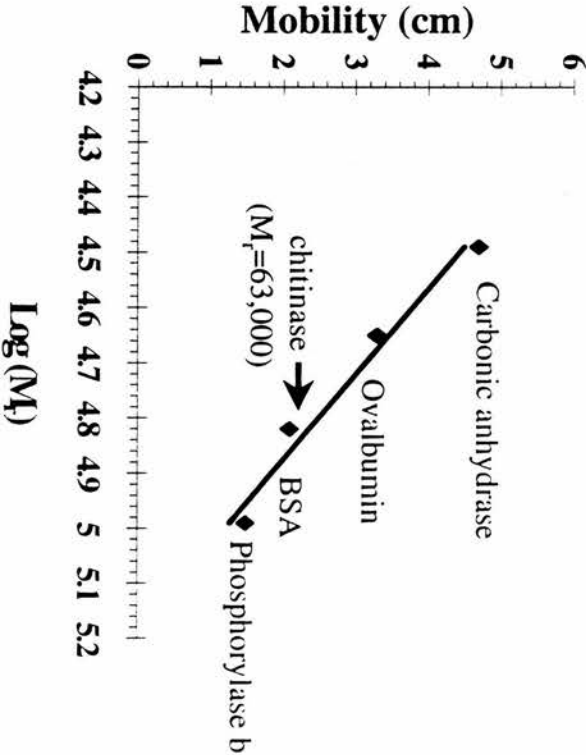
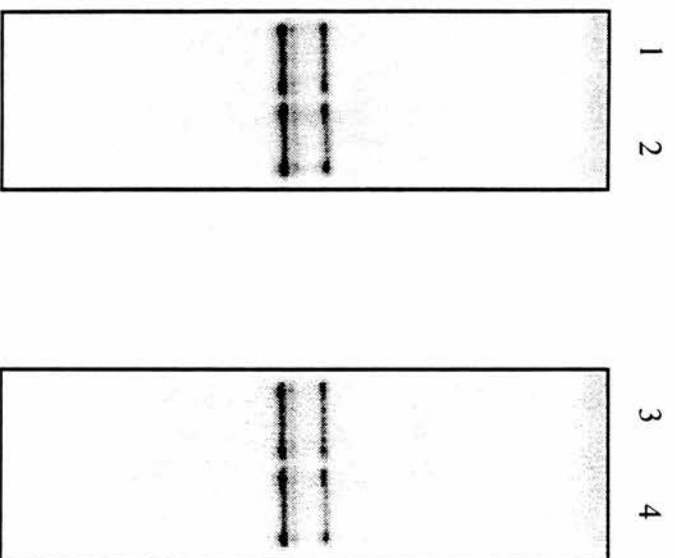


Figure 4.8 Analysis of chitinase activity under non-denaturing electrophoresis conditions

Samples (15 μ l) obtained from the Mono Q FPLC step were loaded onto two non-denaturing acrylamide gels. After electrophoresis, one gel was Coomassie stained (lanes 1 and 2), while the other gel was cut in half and stained separately for chitinase activity towards 4-MU- β -[GlcNac]₂ and 4-MU- β -[GlcNac]₄ prepared in 20 mM potassium phosphate, pH 7.0. After incubation in the dark for 5 min with gentle shaking, the fluorescent bands of 4-methylumbelliferone released were viewed under UV light (312 nm). Lanes 3 and 4 show three protein bands that reacted to 4-MU- β -[GlcNac]₂ and 4-MU- β -[GlcNac]₄ substrates, respectively.



4.4 Partial *N*-Terminal Amino Acid Sequence of *V. carchariae* Chitinase

Once the purified chitinase from *V. carchariae* was obtained, its partial *N*-terminal amino acid sequence was determined. A Mono Q fraction that showed a single protein band on SDS-PAGE was concentrated and subjected directly to Edman degradation on an Applied Biosystems 477A microsequencer as described previously in section 2.2.14. From 20 sequencing cycles performed, only 16 *N*-terminal amino acids could be examined with only 10 amino acids were clearly identified. The sequence was found to be A P T A P S (**I/D D/P M/V**) Y G (**S/M**) N (**N/A**) L Q (brackets indicate cycles in which the interpretation of the sequence was ambiguous; higher yield detected in the ambiguous amino acids are in bold). The initial yield and repetitive yield between A4 (30 pmol) and L15 (14 pmol) were calculated to be 96 pmol and 95%, respectively.

4.5 Production of Polyclonal Antibodies against *V. carchariae* Chitinase

Polyclonal antibodies were produced in order to screen the *V. carchariae* genomic DNA library using an immunological method. The antibodies were therefore raised against purified *V. carchariae* chitinase obtained from the Mono Q FPLC step. Immunisation was performed in a rabbit, and blood collected after the end of the immunisation was tested for its titre and specificity.

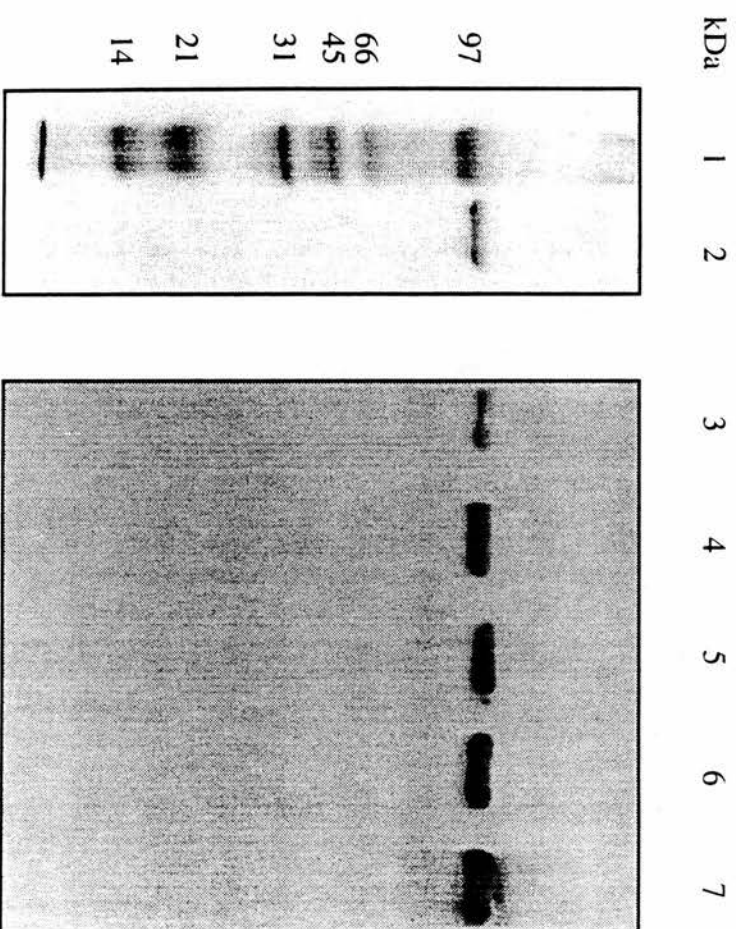
4.5.1 Determination of Titre of Anti-chitinase Serum

One week after an injection of the chitinase (emulsified with TiterMax adjuvant as detailed in section 2.2.11), the titre of anti-chitinase serum produced was tested to be high enough, so that the normal boosting in the following week to increase titre of the antibodies was not required. The titre of the anti-chitinase serum one month after injection was examined. The results in Figure 4.9 show Western blotting analysis of the purified chitinases when different dilutions of the antiserum from 2,500 to 20,000 times were used. It was observed that the anti-chitinase serum reacted with the

Figure 4.9 Determination of titre of anti-chitinase serum

Samples (approx. 10 µg each) obtained from Mono Q FPLC step were applied on 10% SDS-PAGE gel. After electrophoresis, the protein bands were transferred to Hyperbond C nitrocellulose membrane and analysed by Western blotting. Coomassie staining is shown in lanes 1 and 2. Western blotting is shown in lanes 3, 4, 5, 6 and 7 with variable amounts of anti-chitinase serum used.

Lane	Sample
1	low molecular weight protein standards
2	the purified <i>V. carchariae</i> chitinase (Mono Q) (10 µg)
3	Western blotting using 1:20,000 serum dilution
4	Western blotting using 1:10,000 serum dilution
5	Western blotting using 1:7,500 serum dilution
6	Western blotting using 1:5,000 serum dilution
7	Western blotting using 1:2,500 serum dilution



purified chitinase and still gave very strong signals of antibody-antigen complexes, even though the serum was diluted up to 20,000 times.

4.5.2 Determination of Specificity of Anti-chitinase Serum

The blood was also tested against crude enzymes of twelve marine *Vibrios* to investigate whether it cross-reacted with any chitinases from different species. The results in Figure 4.10 show that the antiserum reacted strongly with a single band of the *V. carchariae* crude enzyme at the position of the chitinase enzyme (M_r 66,000). Even though the antiserum cross-reacted strongly with the multiple bands of *V. alginolyticus* 283 crude enzyme and weakly with two protein bands of *V. campbellii* crude enzyme, there was no cross-reaction detected with crude enzymes of the other species tested.

4.6 Carbohydrate Analysis

Paper chromatography and high-voltage paper electrophoresis at pH 2 were performed to prove that smaller chitin fragments broken down by the action of the chitinase at the chitin affinity purification step still remained bound to the protein molecules. After acid hydrolysis, small volumes of protein samples (freshly prepared dialysed CA fraction) were applied on to Whatman papers. Chromatography or electrophoresis was performed as described previously in materials and methods (sections 2.2.9.3 and 2.2.9.4). Results obtained from paper chromatography (Figure 4.11) and high-voltage electrophoresis (Figure 4.12) showed the presence of glucosamine in the CA fraction. The total reducing sugar content of the CA fractions was assayed by the PAHBAH method. After incubation of freshly prepared CA fraction at 30 °C at different times from 1 h to 21 h, followed by ultrafiltration, carbohydrate contents in the retentates and the filtrates were measured. Results in Figure 4.13A indicate an increase of carbohydrate content of the protein in the filtrate fractions from 2.5% (w/w) to 18% (w/w) over 21 h, while Figure 4.13B indicates a decrease in

Figure 4.10 Determination of specificity of anti-chitinase serum

Three parts of concentrated crude samples of the growth media (about 50 µg) from twelve different marine *Vibrios* were mixed with one part of sample buffer. After boiling for 3 minutes, the samples were applied on to a SDS-PAGE gel. After electrophoresis, the protein bands were transferred to Hyperbond C nitrocellulose membrane and analysed by Western blotting with 1:5,000 dilution of serum used.

Lane	Sample
1	<i>V. alginolyticus</i> 283
2	<i>V. alginolyticus</i> 284
3	<i>V. campbellii</i>
4	<i>V. carchariae</i>
5	<i>V. harveyi</i>
6	<i>V. fischeri</i>
7	<i>V. diazotrophicus</i>
8	<i>V.aestuarianus</i>
9	<i>V. natriegens</i>
10	<i>V. pelagius</i>
11	<i>V. gazogenes</i>
12	<i>V.nereis</i>

1 2 3 4 5 6 7 8 9 10 11 12

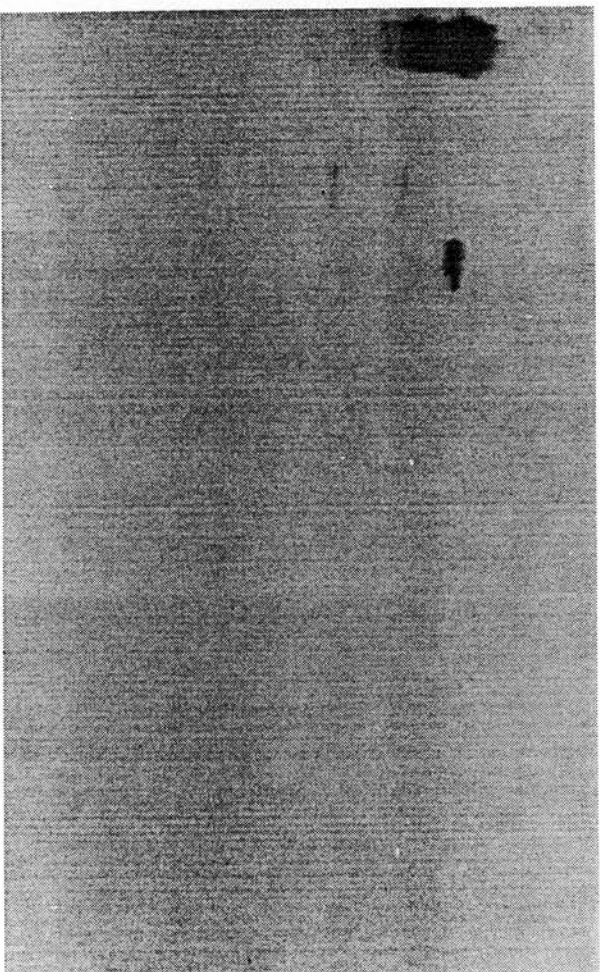


Figure 4.11 Detection of glucosamine in the CA fraction by paper chromatography

Freshly prepared CA fraction (1ml) was hydrolysed at 120 °C for 2 h with 2 M TFA, then acetylated at 100 °C for 1 h with acetic anhydride/pyridine (1:1 v/v) and treated with 2 M TFA as before to complete the hydrolysis. Samples (30 µl) were taken for paper chromatography using a butanol/acetic acid/water solvent (12:3:5 by vol.), with detection by silver nitrate staining.

Lanes	Sample
1	lysine standard
2	chitin, unhydrolysed
3	chitin, hydrolysed
4	glucosamine, unhydrolysed
5	glucosamine, hydrolysed
6	CA fraction, unhydrolysed
7	CA fraction, hydrolysed
8	CA fraction + glucosamine, hydrolysed
9	CA fraction + <i>N</i> -acetylglucosamine, hydrolysed
10	CA fraction + chitin, hydrolysed

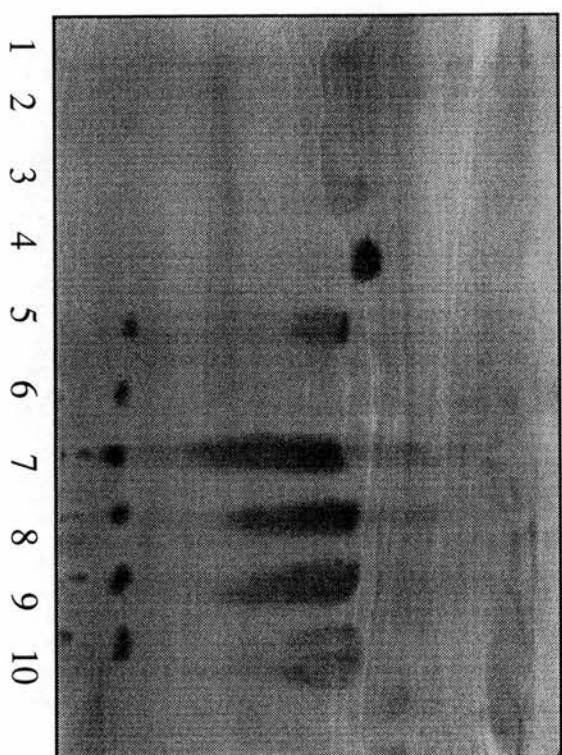
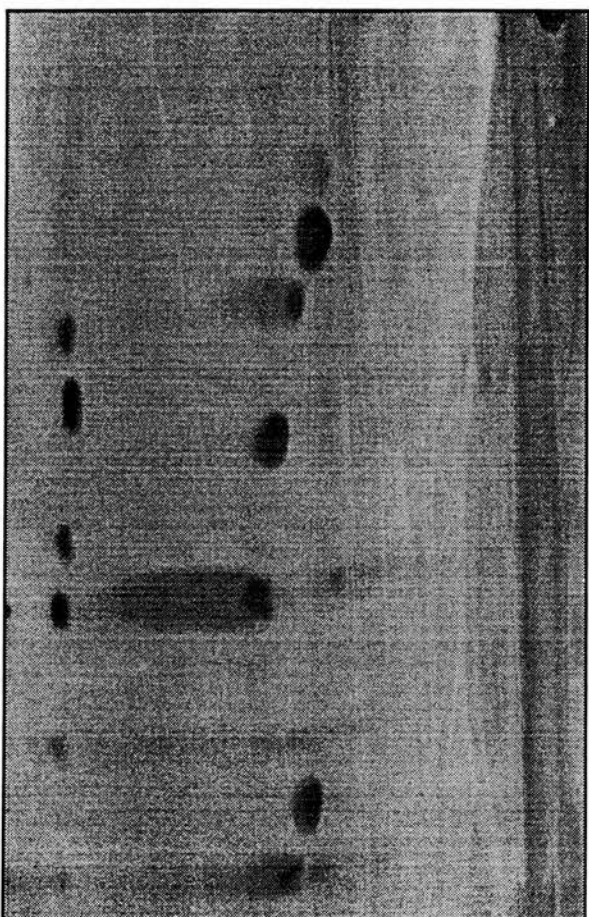


Figure 4.12 Detection of glucosamine in the CA fraction by high-voltage paper electrophoresis

Preparation of samples was carried out in the same manner as for paper chromatography, but electrophoresis was performed under 3 kV for 1 h at pH 2 using a buffer containing formic acid/acetic acid/water (1:4:45 by vol.). After electrophoresis, the chromatogram was stained by silver nitrate.

Lane	Sample
1	methylene blue standard
2	chitin, unhydrolysed
3	chitin, hydrolysed
4	glucosamine, unhydrolysed
5	glucosamine, hydrolysed
6	<i>N</i> -acetylglucosamine, unhydrolysed
7	<i>N</i> -acetylglucosamine, hydrolysed
8	CA fraction, unhydrolysed
9	CA fraction, hydrolysed
10	chitin + BSA, unhydrolysed
11	chitin + BSA, hydrolysed
12	glucosamine + BSA, unhydrolysed
13	glucosamine + BSA, hydrolysed

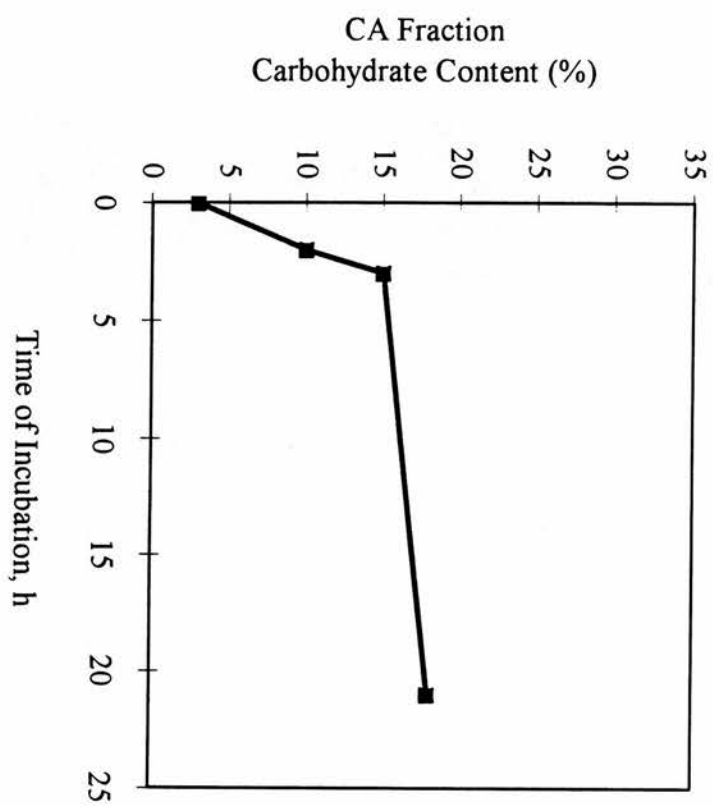


1 2 3 4 5 6 7 8 9 10 11 12 13

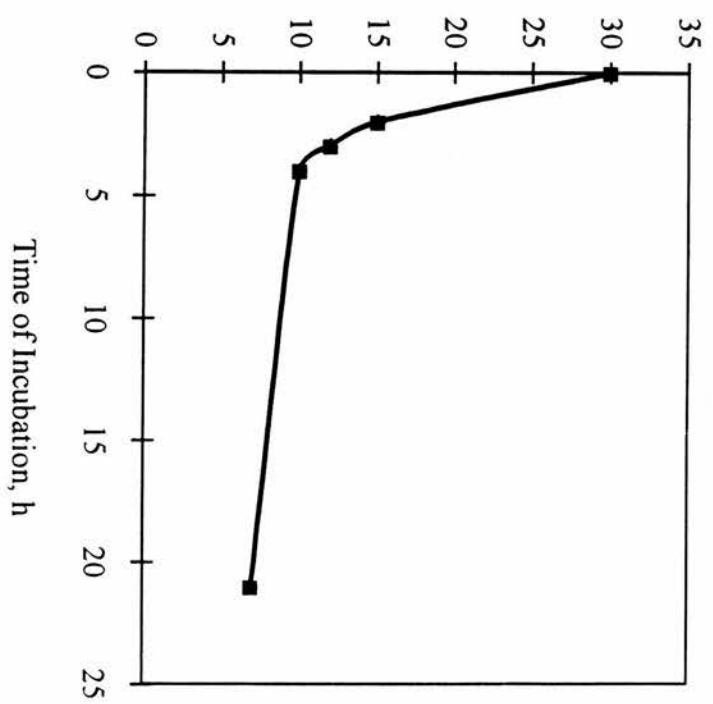
Figure 4.13 Determination of total carbohydrate content of the CA fraction

Freshly prepared CA samples (1 ml) after incubation at 37 °C for different times from 1 h to 21 h followed by ultrafiltration through a Viva spin membrane (10,000 molecular weight cut off, 20 min at 4,000 rpm at 4°C. The ultrafiltered retentates and filtrates were hydrolysed as before. Total reducing sugar was measured by the PAHBAH method. A) Detection of carbohydrate content in the filtrates. B) Detection of carbohydrate content retained on the filter.

A)



B)



carbohydrate content of the protein retained in the filter from 30% (w/w) to 7% (w/w).

4.7 Conclusions and Discussion

4.7.1 Attempts to Develop a Purification Scheme for *V. alginolyticus* Chitinase

Three different purification steps; ammonium sulphate precipitation, ion-exchange chromatography on DEAE-Sephadex A-50, and affinity chromatography on chitin, were tested in order to achieve purification of a chitinase from *V. alginolyticus*. It is common to begin purification with ammonium sulphate precipitation especially when the starting sample (normally crude enzyme) contains a complex mixtures of proteins and other biomolecules i.e. lipids and carbohydrates. This is because the step normally shows an efficient removal of huge amounts of protein. Carbohydrate contamination in protein samples can also be removed by this method. Moreover, compared with general chromatography procedures, the step can be conveniently completed in a short period of time. In this study, ammonium sulphate precipitation appeared to be a useful step. It showed that more than half the total proteins in the sample were removed, while almost total chitinase activity was recovered.

Use of ion-exchange chromatography has been described in the purification of chitinase from various sources (Monreal and Reese, 1968; Tsujibo *et al.*, 1992; Hiraka *et al.*, 1997; and Wang and Chang, 1997). It was found in this study that most of the chitinase was fractionated from unbound or less weakly bound proteins. The step was shown to be useful as it provided an additional purification. Moreover, the overall yield and specific activity obtained after these two purification steps were much higher than reported in previous work (Monreal and Reese, 1968; Tsujibo *et al.*, 1992; Hiraka *et al.*, 1997; and Wang and Chang, 1997).

Chitin affinity was tested and it was found that chitinase had a very high affinity towards chitin. The enzyme bound so tightly to chitin molecules that it could not be eluted with any mild conditions tested. Furthermore, it was found that *N*-acetylglucosamine could not be used to give a specific elution. Explanations from previous work ((Monreal and Reese, 1968; and Soto-Gil and Zyskind, 1984) suggested that *N*-acetylglucosamine in nature is not a real product of chitinase action. On the contrary, enzymatic degradation of chitin appeared to occur in two major steps, which were similar in both prokaryotes and eukaryotes. An endochitinase reduced the chitin polymer to oligomers and dimers, which were subsequently degraded to monomers by the action of chitobiase. Even though affinity binding of chitinase to chitin was disrupted when acetic acid or guanidine HCl were applied, these were likely to be too strong conditions to be used as they basically destroyed the higher molecular structure of the protein, resulting in a complete loss of the enzyme function.

4.7.2 Purification of Chitinase from *V. carchariae*

Before beginning the purification of *V. carchariae* chitinase, the effect of chitin on chitinase expression was determined in order to optimise the initial yield of the enzyme. The result showed that the highest activity of chitinase was observed when the cells were grown in the medium containing 2.5% (w/v) of swollen chitin. This corresponded to a study of Huang (Huang *et al.*, 1996) and of Woo (Woo *et al.*, 1996). They found that production of chitinase reached its maximum when colloidal chitin (2% (w/v) and 3% (w/v), respectively) was added into the growth media. The activity tended to decrease when amounts of chitin substrate higher than 3% was added. However, the mechanism of chitin on chitinase expression at the molecular levels remains unclear.

A purification scheme for *V. carchariae* chitinase comprised three major steps, including chitin affinity (batch-wise), size-exclusion chromatography on Sephacryl S200 HR, and fplc on Mono Q anion-exchanger. The reason for using a batch-wise

procedure for chitin affinity is that performing the experiment in a column was too cumbersome. Chitin is not as homogeneous as commercial resins, so that it was impossible to obtain a well-packed particulate chitin column, and also to avoid air bubbles while the column was running. Another problem encountered was that the volume of chitin packed in the column decreased with time after the sample containing chitinase was applied due to action of the enzyme. It was also found in this study that purification at the chitin affinity step could not be done as quickly as possible. This was because of effect of chitin attached on the protein molecules, thus gave complication particularly in size heterogeneity.

However, chitin affinity chromatography appeared to be an efficient purification step. Most of the proteins applied did not bind to the column but were removed during washing steps. Apart from chitinase, a number of proteins were found to bind non-specifically but tightly to chitin molecules. In order to elute chitin-bound proteins including chitinase, only guanidine HCl seemed to be most suitable among many conditions tested, even though only a very slight chitinase activity (about 3%) remained when 6 M guanidine HCl was used. However, a decrease in concentration of guanidine HCl for elution from 6 M to 2 M strikingly increased the yield of chitinase. Moreover, it should be underlined that the enzyme activity remained after the chitin affinity step was a combination effect of guanidine elution and guanidine denaturation. In other words, guanidine HCl helped release the protein molecules from the chitin affinity, it also simultaneously caused denaturation of the eluted protein in the solution. Therefore, the concentration of guanidine HCl for protein elution should be optimised to obtain the highest yield after chitin affinity step.

Gel filtration on Sephacryl-S200 HR was a useful step for the removal of higher molecular weight proteins that were not removed by chitin affinity. However, some proteins that were similar in size to chitinase could not be removed by this step, and this was overcome at the following Mono Q fplc step.

It was noticed that the overall yield obtained was relatively low especially at the gel filtration step. This could be explained that loss of enzyme activity occurred after chitin affinity step when the protein solution was left standing for a few days at room temperature in order to complete the action chitinase on chitin molecules. It was found that incomplete digestion and remaining chitin products on the enzyme molecules caused difficulties in protein purification. The explanation was supported by another experiment that the higher reproducible yield (approx. 18%) (data not shown) was obtained when further steps after chitin affinity were performed at once.

Based on the molecular weights obtained from gel filtration and SDS-PAGE, *V. carchariae* chitinase comprised a single subunit. The subunit molecular weight (63,000) showed extensive similarities with chitinases isolated from various marine bacteria, for example, *Vibrio fischeri* strain COT-A136 (M_r 63,000) (Fukasawa *et al.*, 1992), *V. alginolyticus* strain TK-22 (M_r 66,000) (Murao *et al.*, 1992), *Alteromonas sp.* strain O-7 (M_r 70,000) (Tsujibo *et al.*, 1992), and *Aeromonas hydrophila* strain H-2330 (M_r 62,000) (Hiraga *et al.*, 1997). According to similarity in their molecular size, these chitinases might be closely related. However, apart from the chitinase from *Alteromonas sp.* strain O-7, there are no amino acid sequences available in database to indicate the relationships of these enzymes.

The chitinase activity was also determined by non-denaturing PAGE. It was discovered that the enzyme that showed single band on SDS-PAGE gave three protein bands on non-denaturing gel. All of which possessed chitinase activity towards the fluorescent substrates (4-MU-[GlcNAc]₂) and (4-MU-[GlcNAc]₄). This might be explained as deamidation in the protein molecules. This situation commonly occurs when protein are exposed to acidic conditions. Heterogeneity in charge and size caused from post-translation processes or even from chitin attachment could also be another explanation for multiple bands of the chitinase on non-denaturing gel.

4.7.3 Partial N-Terminal Amino Acid Sequence

The partial *N*-terminal sequence of *V. carchariae* chitinase showed similarity with the *N*-terminal sequences of ChiA of *Serratia marcescens* (Chia_serma) (Jones *et al.*, 1986), ChiA of *Alteromonas sp.* strain O-7 (Chia_altso) (Tsujibo *et al.*, 1992), and ChiA of *Enterobacter agglomerans* (Chia_entag) (Chernin *et al.*, 1997). A comparison of the 16 *N*-terminal amino acids of the three chitinases is illustrated as follows:

<i>V. carchariae</i> chitinase	A	P	T	A	P	S	<u>I</u>	<u>D</u>	<u>M</u>	Y	G	<u>S</u>	N	<u>N</u>	L	Q
Chia_altso	A	P	S	T	P	T	L	D	W	Q	P	Q	Q	Y	S	F
Chia_serma	A	P	G	K	P	T	I	A	W	G	N	T	K	F	A	I
Chia_entag	A	P	G	K	P	S	T	A	S	G	P	T	K	F	A	I
			*	*				*								

* Represents where the amino acids are identical.

The underlined amino acids residues were ambiguous, but they gave higher yields over the other residues (see section 4.4). Therefore, they were chosen for the amino acid comparison.

Based on the *N*-terminal amino acid sequence comparison shown above and some other physical properties mentioned previously, it is likely that the chitinase isolated from *V. carchariae* belongs to the same group of the enzymes. However, there is no relation between the sequence of *V. carchariae* and a published sequence of *V. harveyi* chitinase. It might be that the chitinase from *V. harveyi* is from a different chitinase group.

4.7.4 Production of Polyclonal Antibodies

Polyclonal antibodies raised against the purified *V. carchariae* chitinase showed a very high titre on Western blotting analysis as a strong signal of antibody-antigen complexes was still observed even if they were diluted up to 20,000 times. Moreover,

among 12 species of marine *Vibrio* tested, the antibodies cross-reacted strongly with only the crude enzyme of *V. alginolyticus* 283 and very slightly with that of *V. campbellii*. This supported similarity of the *V. alginolyticus* 283 and *V. carchariae* enzymes. The multiple protein bands from crude enzyme of *V. alginolyticus* 283 and the two protein bands from crude enzyme of *V. campbellii* that recognised the anti-chitinase serum were thought to belong to chitinases expressed from the multiple gene system in the bacteria. This result supports the idea of the multiple forms of chitinase produced by the bacteria as discussed previously in chapter 3 (section 3.4.2). No cross-reaction of the anti-chitinase serum to crude enzyme of *V. harveyi* was observed, even though the bacterium was proved to secrete some chitinases (as shown in chapter 3, Table 3.2). This clearly indicated no similarity between chitinases of *V. harveyi* and the chitinase of *V. carchariae*. This result also corresponded to the amino acid sequence comparison between the two bacterial species as noted in the previous section. Even though the anti-chitinase serum produced from the purified chitinase of *V. carchariae* cross-reacted with some proteins of other bacteria, only a single protein was recognised in the crude enzymes of the same species. The protein band appeared to belong to the chitinase that was purified and used to prepare the antibodies. This is an apparent indication that the antibodies raised were highly specific to chitinase and should be suitable for further use, especially for screening the genomic library of *V. carchariae*.

4.7.5 Carbohydrate Analysis

Even though the initial strategy was to carry out each purification step as quickly as possible to minimise proteolysis and loss of activity, it was eventually realised that sample heterogeneity was reduced by proceeding at a more leisurely pace. It seemed likely that the heterogeneity might have been caused by variable amounts of chitin fragments bound tightly to the chitinase, and that these were subsequently released into small soluble products by the action of the chitinase. This hypothesis was confirmed by qualitative carbohydrate analysis by presence of glucosamine, which is a major product of acid-hydrolysis reaction, in the protein sample. A decrease of

carbohydrate content found in the retentates was also correspond with an increase of carbohydrate content in detected the filtrates.

Chapter 5

Development of Nucleic Acid Probes for Genomic DNA Library Screening

5.1 Introduction

Three different approaches have been employed in recent studies in order to isolate genes encoding chitinases from various bacterial species:

1) Conventional method on chitin agar plates

The chitin plate assay is achieved by the development of clear zones around stab sites of colonies producing chitinase. The clear zones are formed by the action of chitinase activity towards chitin substrates in the agar medium. Particular advantages of the method are that clones expressing active chitinase are determined and it is also considered to be the most convenient method.

2) Immunological methods using monoclonal or polyclonal antibodies

The immunological methods exemplified for screening of genomic DNA libraries for chitinase genes are based on the specific binding between anti-chitinase serum, which is raised against the purified enzyme, and clones expressing a recombinant chitinase. This method has an advantage in that genes that do not confer any selectable property on the host can be detected, but it does require specific antibodies.

3) Nucleic acid hybridisation methods.

Screening procedures, which rely on nucleic acid hybridisation, are general in application and powerful. The principle of the method is specific hybridisation between nucleic acid probes and clones carrying a chitinase gene. DNA or cDNA fragments containing a chitinase gene are normally obtained by PCR amplification techniques. Nucleic acid fragments will be labelled non-radioactive or radioactive and used as nucleic acid probes. Using these procedures, it is now considered possible to easily isolate any gene from virtually any organism, but the problem of gene isolation is a problem of obtaining a suitable probe.

To isolate the gene encoding chitinase from *V. chachariae*, it was necessary to determine the best screening method for the genomic DNA library. In this study, all the three methods mentioned above were evaluated, but initial attempts were made by focusing on DNA probes prepared by PCR amplification using genomic DNA of *V. carchariae* as DNA template with degenerate oligonucleotide primers, designed from N-terminal amino acids of the purified *V. carchariae* chitinase.

5.2 Design of Degenerate Oligonucleotide Primers

Three sets of degenerate oligonucleotides, designated P1, P2, and P3, were designed using a computer program called "BACKTRANSLATE" (Figure 5.1). Reversed translation was made on the basis of minimal degeneracy and codon bias and with respect to highly expressed codon usage (better than 10% usage in *E. coli*).

5.3 Attempted Development of Nucleic Acid Probes

PCR amplification was performed using three sets of the oligonucleotides synthesised in mixtures as described before as well as four universal primers including: T3 (5'-AATTAACCCTCACTAAAGGG-3'), T7 (5'-TAATACGACTCACTATAGGG-3'), M13 (5'-TGTAACACGACGGCCAGT-3'), M13(rev) (5'-CAGGAAACAGCT

ATGACC-3') as flanking nucleotides. *EcoR* I partial digests of genomic DNA of *V. carchariae*, which were ligated into pBluescript II KS (-) vector, were used as DNA templates. The possible orientations of insert obtained by PCR reaction using a combination of the primer mixtures are illustrated in Figure 5.2

Target *N*-terminal Amino Acid Sequence

N-----**M A P T A P** S D P **V Y G S/M N A L** Q A L D R/P/I-----*C*

region1

region2

Region1 : Met Ala Pro Thr Ala Pro

AUG GCU CCG ACC GCU CCG

A U A

G G

Proposed degenerate oligonucleotides, "P1"

5'-ATG GC(AGT) CCG AC(CT) GC(TAG) CCG-3'

Region2 : Val Tyr Gly Ser/Met Asn Ala Leu

GUU UAC GGU NNN AAC GCU CUG

A U C A

G G

Proposed degenerate oligonucleotides, "P2"

5'-GT(TAG) TA(CT) GG(TC) III* AAC GC(TAG) CT-3'

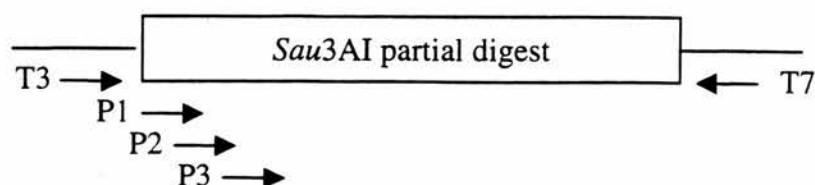
I* Represents Ionosine

Proposed degenerate oligonucleotides, "P3"

5'- GT(TAG) TA(TC) GG(TC) ATG AAC GC(TAG) CT-3'

Figure 5.1 Illustration of two regions of *N*-terminal amino acids of the *V. carchariae* chitinase where the primers P1, P2, and P3 were designed.

A) Forward direction



B) Reverse direction

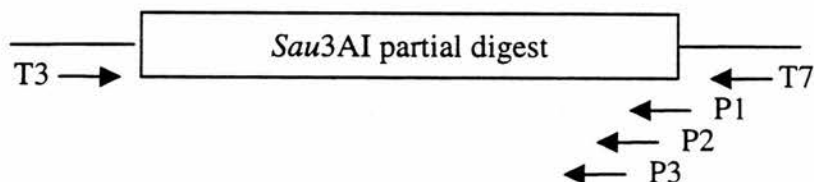


Figure 5.2 Illustration of *Sau3A* I partial digests that were used as DNA templates in the PCR experiment using a combination of the degenerate oligonucleotide primers (P1, P2, or P3) and the flanking primers (T3, T7, M13, or M13(rev)) as DNA primers. Both possible orientations that generated PCR products are indicated. A) forward direction; B) reverse direction.

5.3.1 Analysis of PCR Products on Agarose Gel

To test which degenerate oligonucleotide primers were suitable to be used in PCR amplification, a combination of upstream primers (P1, P2, or P3) with universal primers (T3, T7, M13, and M13(rev)) were tried (see Figure 5.2). It was found that some amplified products were observed with a combination of P1 and T3, and of P1 and T7 used in the first round PCR. In order to obtain higher yields of such products, second round PCR was performed using the products of the first round PCR reaction as DNA templates and with the same set of primers, but no product was detected at all (Figure 5.3).

When the PCR reaction was carried out with P1 and M13(rev), a major band of PCR products at about 800 bp, designated "P1M13(rev)", was observed, but no amplified product was detected when the PCR reaction was carried out with P1 and M13 (Figure 5.4).

In addition, no amplified product was observed when the reactions were performed with combinations of P2 or P3 with all the universal primers tested (data not shown).

5.3.2 Manual DNA Sequencing

It was concluded at this stage that amplification using P1 and M13(rev) appeared to provide convincing PCR products. Therefore, terminal nucleotide sequences of P1M13(rev) were investigated in order to confirm that authentic products were obtained before they were further used as DNA probes. Manual DNA sequencing was performed according the Sanger method (see section 2.2.18.7). PCR products "P1M13(rev)" were excised and purified on agarose, then subcloned into pGEM-T vector and electrotransformed into *E. coli* XL1 Blue. Double digests of DNA plasmid with *Pst* I and *Nco* I showed that among 10 transformed *E. coli* colonies picked, eight colonies contained DNA inserts (Figure 5.5). For DNA sequencing analysis, five

Figure 5.3 Gel electrophoresis of PCR products (P1T3 and P1T7)

First round PCR amplification was performed using *EcoR* I partial digest of genomic DNA of *V. carchariae*, ligated into pBluescript II KS (-) vector, as templates. P1 and T3 or T7 were used as DNA primers. Second round PCR was performed using first round PCR reaction products as templates with the same set of primers. Conditions used in PCR reaction were outlined in section 2.2.18.4.

Lane	Sample
1	1 kb ladder DNA marker
2	PCR reaction without primers (primer control)
3	PCR reaction without plasmid (vector control)
4	first round PCR with P1 and T3 primers
5	first round PCR with P1 and T7 primers
6	second round PCR with P1 and T3 primers
7	second round PCR with P1 and T7 primers

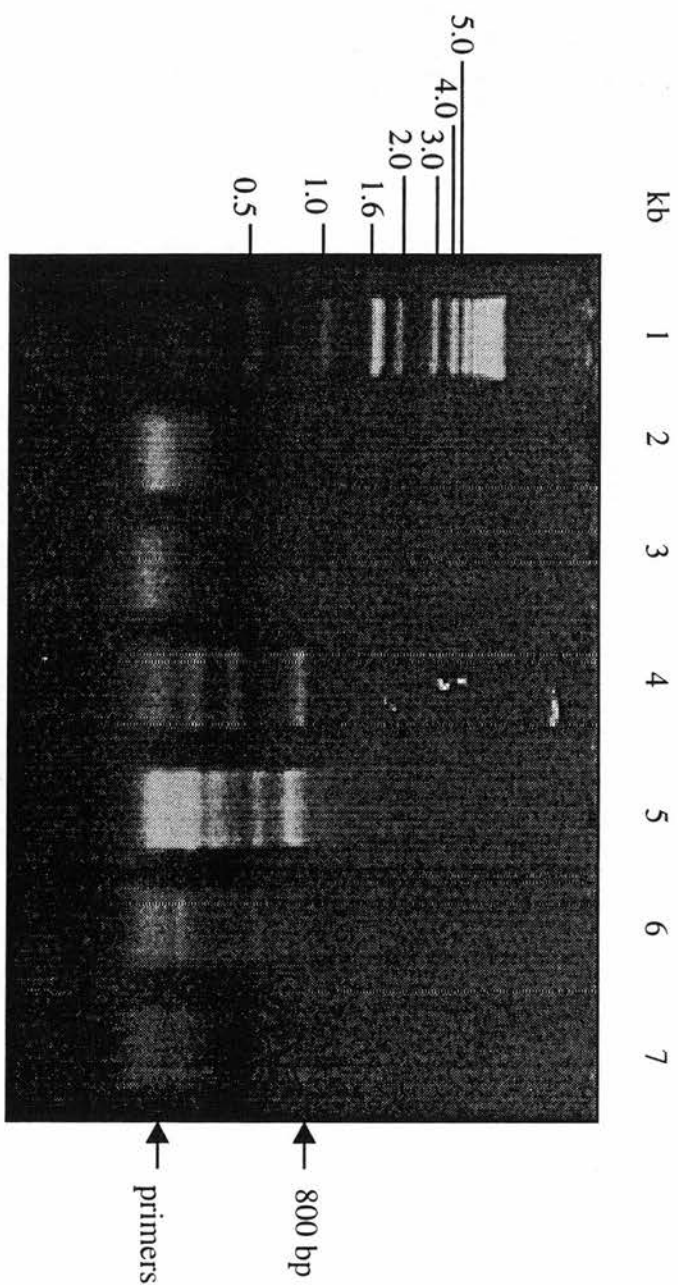


Figure 5.4 Gel electrophoresis of PCR products (P1M13 and P1M13(rev))

PCR amplification was performed using *EcoR* I partial digest of genomic DNA of *V. carchariae*, ligated into pBluescript II KS(-) vector as templates. P1 and M13 or M13(rev) were used as DNA primers. Conditions were as outlined in section 2.2.18.4.

Lane	Sample
1	1 kb ladder DNA marker
2	PCR amplification with P1 and M13 primers
3	PCR amplification with P1 and M13(rev) primers

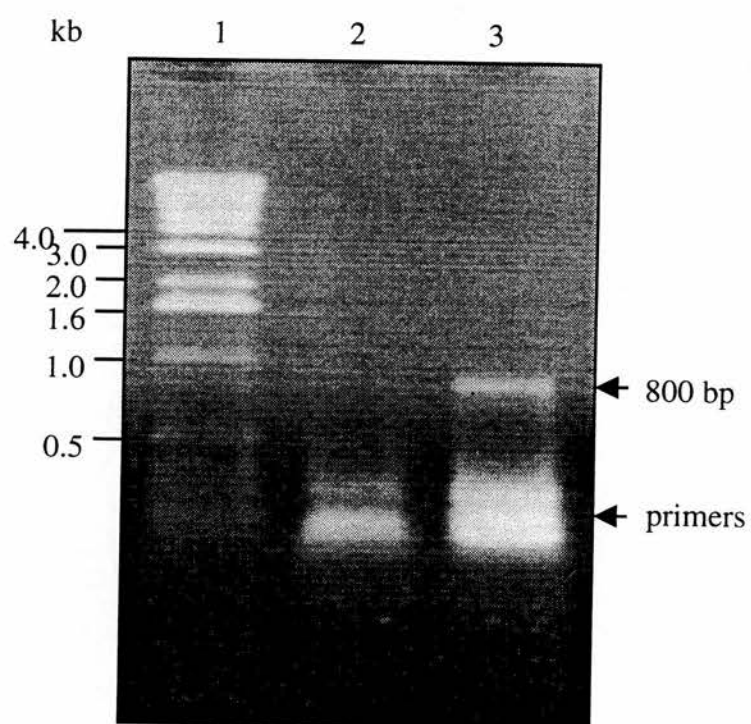
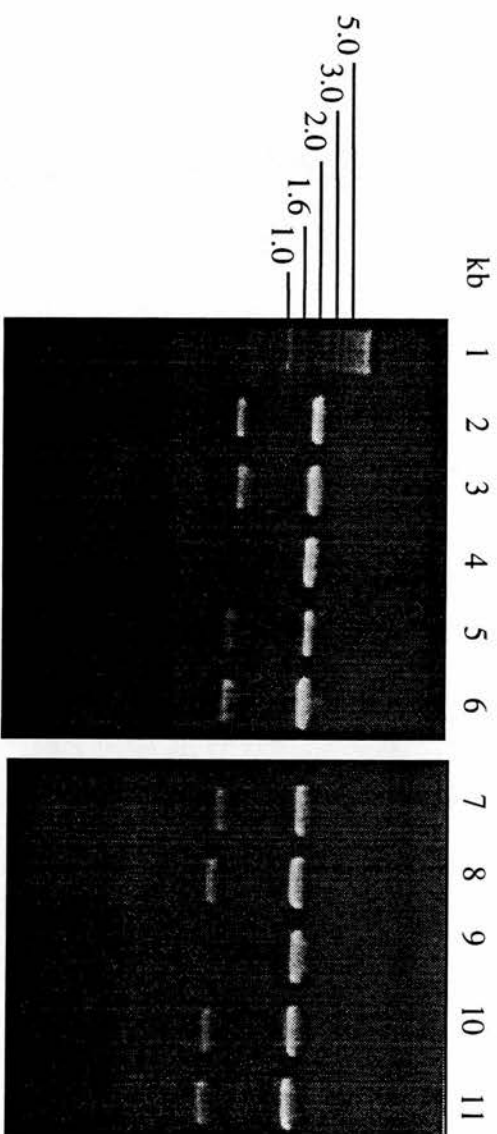


Figure 5.5 Gel electrophoresis of p-GEM-T clones carrying PCR products

PCR products of P1M13(rev) were purified on agarose gel, then ligated into p-GEM-T vector. After introduction of recombinant plasmids into *E.coli* XL1 Blue, 10 white colonies obtained on LB agar plates containing ampicillin, X-gal and IPTG were picked and DNA plasmids were prepared by the small-scale preparation method. DNA plasmids were double digested with *Pst* I and *Nco* I according to conditions outlined in section 2.2.18.6 and analysed on 1% agarose gel.

Lane	Sample
1	1 kb ladder DNA marker
2-11	ten white colonies tested



transformants that contained plasmids carrying DNA inserts were prepared and used as DNA templates. Partial nucleotide sequences at both ends, namely pGEM1, pGEM2, pGEM3, pGEM4, and pGEM5, were investigated with a forward primer T7 and a reverse primer; SP6 (5'-ATTAGGTGACACTATA-3') or M13 (rev). The physical map of pGEM-T containing PCR inserts is illustrated in Figure5.6.



Figure 5.6 A physical map of pGEM-T vector carrying DNA insert prepared from partial digests of genomic DNA of *V. carchariae* and used as templates for DNA sequencing analysis.

Sequencing results obtained (see diagram below) showed that the clones carrying 800 bp-PCR insert were different (except pGEM2 and pGEM5). Moreover, it was also found that most the nucleotide sequences at both upstream and downstream encoded identical amino acids (M A P T A P), indicating non-specific PCR products were amplified. Approximately 120 nucleotides were sequenced in each clone, but only the first 36 nucleotides are shown in the diagram below.

pGEM1-T7

5'-GGGATT ATG GCG CCG ACT GCC GCA CAA CAG AAT ACC

Amino acid: M A P T A A Q Q N T

pGEM1-M13(rev)

3'-GTG ATT ATG GCG CCG ACT CGC ACC GTT TTC AAT GTG

Amino acid: M A P T A T V F N V

pGEM2-T7

5'-GGG ATT ATG GCG CCG ACT GCA CCG TAC GAA TAC GTC...

Amino acid: M A P T A P Y E Y V

pGEM2-M13(rev)

3'-GTG ATT ATG GCG CCG ACT GCG CCG TTC ACG AAG TTG...

Amino acid: M A P T A P F T K L

pGEM3-T7

5'- GGG ATT ATG GCA CCG ACT GCG CCG CAC AAC AGA ATA...

Amino acid: M A P T A P H N R I

pGEM3-M13(rev)

3'-GTG ATT ATG GCG CCG ACT GCA CCG TTT TCA ATG TGA...

Amino acid: M A P T A P F S M Ter.

pGEM4-T7

5'-GGG ATT ATG GCT CCG ACT GCG CCG CAC AAC AGA ATA...

Amino acid: M A P T A P H N R I

pGEM4-M13(rev)

3'-GTG ATT ATG GCG CCG ACT GCA CCG TTT TCA ATG TGA...

Amino acid: M A P T A P F S M Ter.

pGEM5-T7

5'GGGATT ATG GCT CCG ACT GCA CCG TAC GAA TAC GTC...

Amino acid: M A P T A P Y E Y V

pGEMT5-SP6

3'-GTG ATT ATG GCG CCG ACT GCA CCG TTT TCA ATG TGA...

Amino acid: M A P T A P F S M Ter.

5.4 Conclusions and Discussion

From this study, the development of nucleic acid probes for the chitinase gene in genomic DNA of *V. carchariae* proved to be unsuccessful. Even though some products (P1T3 and P1T7) were amplified, it was realised that the results obtained were not reliable, as indicated from unrecovered products in the second round PCR with the same set of primers used. Although a very intense band at 800 bp was observed when PCR amplification was performed with P1 and M13(rev), DNA sequencing results obviously showed that the band contained heterogeneous DNA fragments and they were more likely to be non-specifically amplified products. This was proved from nucleotide sequences that encoded identical amino acids at both ends of each DNA fragment. Having no products or non-specific hybridisation detected could be explained as a result of poor quality of oligonucleotides used as PCR primers. Although amino acid regions that contained minimal codon degeneracy were chosen, the primers were synthesised as a mixture of oligonucleotides and each primer had high degeneracy number (P1 had 18 degeneracies, P2 and P3 had 36 degeneracies).

Moreover, the *N*-terminal amino acid region that was chosen for design of P2 and P3 contained some amino acids that were ambiguous. This is clearly indicated in *N*-terminal amino acid sequence investigated in chapter 4 (section 4.4). Moreover, when considered the amino acid sequence translated from DNA sequence of *V. carchariae* gene in chapter 6 (Figure 6.20), it showed that the amino acid region that was chosen for design of P2 and P3 contained two wrong amino acids. The selected valine at the residue no 9 was found to be methionine, and alanine at the residue no 14 was found to be arginine in the amino acid sequence of *V. carchariae* converted from the nucleotide sequence. So, this clearly explained unsuccessful PCR using the two primers.

Chapter 6

Isolation of A Gene Encoding Chitinase from *Vibrio carchariae*

6.1 Introduction

A key approach to understanding the genetic basis for the production of chitinases has been the isolation and characterisation of the genes encoding them. In marine bacteria, genetic manipulation of the chitinase gene using DNA technology has been well established in *Alteromonas sp.* The chitinase enzyme was purified from a marine bacterium *Alteromonas sp.* strain O-7, and its partial amino acid sequence were determined by Tsujibo *et al.* (1992). Its gene cloning, sequencing and expression was reported in the following year (Tsuijibo *et al.*, 1993). Their work suggested that a product produced by recombinant DNA was accumulated in the periplasmic space while the product produced by *Alteromonas sp.* was secreted into the culture medium. It was also found that two chitinases with different molecular weights were products of a single chitinase gene since these two proteins showed almost the same enzymic properties and the *N*-terminal sequences of the two enzymes were identical. The forms of enzymes with different molecular sizes were suggested to be a result of partial proteolysis occurring in the *C*-terminal region. From nucleotide sequence analysis, a single open reading frame was found to encode a protein consisting of 820 amino acids with a predicted molecular weight of 87,341. A putative ribosome-binding site, promoter, and signal sequence were identified. The deduced amino acid sequence of the cloned chitinase showed sequence similarity with ChiA and ChiB from *S. marcescens*, indicating a common ancestor of these organisms.

Cloning and nucleotide sequence of the gene-encoding Chi II from a different strain of *Aeromonas* sp were studied by Ueda *et al.* (1994). A gene encoding Chi II from *Aeromonas* sp. no 10S-24 was transformed into *E. coli* DH5 α using pUC19 as a vector. Deletion analysis and nucleotide sequence data of plasmids constructed by digestion of the plasmid containing the *Aeromonas* DNA fragment showed that the 3.0-kb fragment contained the full-length chitinase structural gene. Nucleotide sequence of the inserted fragment showed an open reading frame consisting of 1,626 base pairs that coded for a protein of 542 amino acids. A typical promotor and Shine-Dalgarno region were located upstream of the initiation ATG codon. The deduced amino acid sequence of the cloned Chi II showed sequence similarity with the chitinase from *Saccharopolyspora erythraea* (26% identity). This result suggested that the chitinase gene of *S. erythraea* and the Chi II gene of *Aeromonas* sp. no 10S-24 are derived from an ancestral chitinase gene. They also observed that the Chi II had Pro-Thr rich domains, consisting of repeat sequences of 47 amino acids, which were not conserved, in other chitinases from bacteria. The repeat sequence of amino acids rich in Pro and/or Thr has been proposed to link discrete functional domains in many cellulases and xylanases (Gilkes *et al.*, 1991).

In 1998, Tsujibo and his colleagues (Tsujibo *et al.*, 1998) reported the characterisation of ChiC from the same bacterial strain (*Alteromonas* sp. strain O-7). Its corresponding gene was investigated, and revealed an open reading frame encoding for a protein of 430 amino acids with a predicted molecular mass of 48 kDa. Alignment of the deduced amino acid sequence demonstrated that ChiC contained three functional domains, the *N*-terminal domain, a fibronectin type III-like domain, and a catalytic domain. The role of the *N*-terminal domain was evaluated to be an independent chitin-binding domain.

The study of a *Vibrio* chitinase was described by Soto-Gil and Zyskind (1984). A library containing DNA from the chitinolytic marine bacterium *Vibrio harveyi* was constructed in *E. coli*. The two clones were capable of expressing chitobiase (which degrades disaccharides only) activity, carrying inserts 5.3 kb and 13.5 kb in length. They also exhibited chitinase activity, indicating a link between the chitinase and

chitobiase genes in *V. harveyi*. *N,N'*-diacetylchitobiose was examined as a strong inducer of both *V. harveyi* chitinases but not of *E. coli* expressed chitinases. A *chi* operon in *V. harveyi* was suggested to consist of genes coding for chitinase, chitobiase, and possibly a permease for *N,N'*-diacetylchitobiose.

A more recent report on *V. harveyi* chitinase study was done by Svityl *et al.* (1997). They reported six separated chitinase genes from *Vibrio harveyi* were cloned and these genes appeared to be unique based on DNA restriction patterns, immunological data, and enzyme activity. Separated chitinase genes expressed in the marine bacterium was suggested to play an important role in efficient utilisation of different forms of chitin and chitin-by products.

Study of genetic control of the chitinolytic enzyme in *Vibrio vulnificus* (Wortman *et al.*, 1986) was performed by transforming chromosomal DNA of *V. vulnificus* into *E. coli* DH1 using plasmid pBR322 as a vector. Endochitinase activity of the insert-bearing clones was determined by liberation of water-soluble products produced by the degradation of [³H]chitin. Chitobiase activity was also detected from the clones carrying chitinase genes. It was found that *V. vulnificus* exported chitinase enzymes into the surrounding medium and produced large clear zones in colloidal chitin overlays. However, the *E. coli* colonies carrying the chitinase genes did not produce the clear zones on plates overlaid with colloidal chitin, suggesting that the chitinase enzymes were not exported by *E. coli* DH1. From enzyme assay using [³H]chitin, chitinase activity was detected in cytoplasmic and membrane fractions, but higher activity was found in the cytoplasmic fraction. They found that transformation of *E. coli* Y10R (*lacY*) with plasmids from chitinase positive clones restored the lactose-positive phenotype, which suggested the presence of a permease associated with chitinase activity. Physical mapping of plasmids containing the chitinase determinants indicated that transcription of chitinase genes in *E. coli* might be initiated at a *V. vulnificus* promotor.

Cloning, expression, purification and characterisation of a different type of chitinase from *V. parahaemolyticus* have been reported (Laine *et al.*, 1988). They found that

one clone in 2,000 produced a clear zone of hydrolysis on particulate chitin agar plates. The subclone carrying a chitinase gene produced the 95 kDa mature chitinase that was stored in both periplasmic and extracellular compartments. The molecular weight of cloned chitinase was found to be the same with the native chitinase secreted by *V. parahaemolyticus*. Deletion analysis study showed that a variant carrying a 2.9 kb DNA insert that was generated from transformant *E. coli* containing 5.4 kb genomic DNA of *V. parahaemolyticus* produced an 11-fold increase in the level of chitinase expression when the orientation of the insert was changed. They also found that the cellular localisation of chitinase was influenced by the orientation of the insert. The minimum insert length necessary for chitinase activity was shown as 1.9 kb. Deletion analysis showed that different deletion variants secreted and accumulated the enzyme in different subcellular locations but the *N*-terminal amino acid appeared to be the same. Some physical characteristics of the mature enzyme were investigated, and the effect of salt concentrations on the enzyme activity was studied.

Fukasawa and colleagues (Fukasawa *et al.*, 1992) studied some properties of a chitinase from a marine luminous *Vibrio* strain COT-A136 isolated from the intestine of fish i.e. native and subunit molecular sizes, pH and temperature optima and stability. However, molecular biology studies of chitinase from this strain have not been done.

The most recent report was on *Vibrio anguillarum* strain KV9001 and *V. parahaemolyticus* strain ATCC17802. Genes encoding chitinases from both bacteria were transformed into *E. coli* (Hirono *et al.*, 1998). They found that the open reading frames of chitinase genes from *V. anguillarum* (*vac*) and *V. parahaemolyticus* (*vpc*) are 1,755 bp and 1,890 bp, respectively. Clear zones produced by *E. coli* colonies carrying chitinase genes indicated that the cloned chitinases were expressed and secreted from *E. coli* cells. It was found that the nucleotide and amino acid sequences of the chitinases were similar to each other. The similarities of DNA and amino acid sequences of *vac* and *vpc* are 69% and 72%, respectively. They also found that *vac* gene was highly prevalent in *V. anguillarum*, and the DNA probe of

vac gene hybridised to *V. alginolyticus* and *Benecke proteolytica* DNA. The DNA probe of the vpc gene hybridised to *V. alginolyticus*, *V. harveyi*, and *V. ordalii* DNA. The molecular studies of chitinases in marine bacteria is summarised in Table 6.1

In this chapter the cloning and sequencing of the gene encoding chitinase from *V. carchariae* is reported. Analysis of the sequence and expression of the recombinant chitinase in *E. coli* is also discussed.

6.2 Preparation of a Genomic DNA Library from *Vibrio carchariae*

6.2.1 Preparation of Genomic DNA for Cloning

The principle steps involved in preparing genomic DNA for cloning are: 1) isolation of genomic DNA from *V. carchariae* culture; 2) partial digestion of DNA and 3) size fractionation. Steps for genomic DNA isolation included SDS-proteinase K treatment followed by treatment with cetyltrimethylammonium bromide (CTAB), then phenol/chloroform/isoamyl alcohol extraction. Prior to beginning the actual cloning experiment, DNA extraction techniques were practised until good quality genomic DNA (minimal smear bands of DNA were observed on agarose gel) was obtained. To prepare genomic DNA for cloning, DNA was partially digested with *Sau3A* I. However, prior to performing large-scale digestion, restriction conditions were optimised on a small scale in order to establish the optimal enzyme concentration to generate a certain size range of DNA fragments (from 4-7 kb). In this study, small-scale reactions containing different amounts of *Sau3A* I from 3.5 mU to 1U were tested. Figure 6.1 shows that very small fragments (less than 1 kb) were generated when DNA was digested with 1U of *Sau3A* I. Incomplete digestion was generated when DNA was digested with less than 50 mU of *Sau3A* I. When 100 mU enzyme was used, DNA fragments between 3->10 kb were generated. In order to maximise the amount of DNA fragments from 4-10 kb, a narrower dilution range of *Sau3A* I from 125 mU to 500 mU were tested. As can be seen in Figure 6.2, the maximum quantity of the desired size range was given when DNA was digested with 125 mU of *Sau3A* I. This amount of enzyme was therefore used to carry out a large-scale

Table 6.1 The summary of chitinase studies in marine bacteria

Bacterial species	General features	References
<i>Alleromonas</i> sp. strain O-7	<ul style="list-style-type: none">- A gene encoding ChiA was expressed in <i>E. coli</i> using pUC18, and sequenced- <i>E. coli</i> produced two active chitinases with different molecular weight (Chi-85 and Chi-78)- The cloned chitinases were stored in periplasmic space- The Chi-78 appeared to be the partial proteolytic product of Chi-85	Tsujibo <i>et al.</i> , 1992 and 1993
<i>Alleromonas</i> sp. strain O-7	<ul style="list-style-type: none">- A gene encoding ChiC was expressed in <i>E. coli</i> using pGEX-5X-3 expression vector, and sequenced- The molecular mass of the cloned chitinase was calculated to be 48 kDa- ChiC contains three functional domains, the N-terminal domain, fibronectin like domain, and catalytic domain	Tsujibo <i>et al.</i> , 1998
<i>Aeromonas</i> sp. no. 10S-24	<ul style="list-style-type: none">- The bacterium produced five chitinases that differ in their N-terminal sequences- A gene encoding Chi II was expressed in <i>E. coli</i> using pUC19, and sequenced- <i>E. coli</i> expressed the precursor protein with Pro-Thr rich domains- The molecular weight of the mature protein (53,140) was only half of the native chitinase (115,00)	Ueda <i>et al.</i> , 1994
<i>Vibrio harveyi</i>	<ul style="list-style-type: none">- Two <i>E. coli</i> constructs carrying DNA inserts from <i>V. harveyi</i> expressed chitinase as well as chitinase activity- A <i>chi</i> operon of <i>V. harveyi</i> was suggested to consist of genes encoding chitinase, chitinase, and a permease	Soto-Gil and Zyskind, 1984

The summary of chitinase studies in marine bacteria (continued)

Bacterial species	General features	References
<i>Vibrio harveyi</i>	<ul style="list-style-type: none">- <i>V. harveyi</i> secreted more than ten chitinases, but six separate chitinase genes were expressed in <i>E. coli</i> using pBluescript II KS(-)- All the cloned chitinase showed unique properties based on DNA restriction patterns, immunological data, and enzyme activity	Svitlyl <i>et al.</i> , 1997
<i>Vibrio vulnificus</i>	<ul style="list-style-type: none">- Genes encoding chitinase were expressed in <i>E. coli</i> using pBR322- <i>E. coli</i> expressed the fusion protein with endochitinase activity- The chitinase activity was detected in cytoplasmic and membrane fractions	Wortman <i>et al.</i> , 1986
<i>Vibrio parahaemolyticus</i>	<ul style="list-style-type: none">- A gene encoded chitinase was expressed in <i>E. coli</i> using pUC18- <i>E. coli</i> expressed the mature 95 kDa chitinase that was stored in both periplasmic and extracellular compartments- Expression and the cellular localisation of the chitinase were influenced by the orientation of the insert.	Laine <i>et al.</i> , 1988
<i>Vibrio anguillarum</i> strain KV9001 and <i>V. parahaemolyticus</i> strain ATCC17802	<ul style="list-style-type: none">- Genes encoding chitinases were expressed in <i>E. coli</i> using pUC118 and pUC119, and sequenced- The two chitinases showed similarities in their nucleotide and amino acid sequences- The DNA probe of <i>V. anguillarum</i> chitinase gene hybridised to <i>V. alginolyticus</i> and <i>Beneckeia proteolytica</i> DNA- The DNA probe of <i>V. parahaemolyticus</i> chitinase gene hybridised to <i>V. alginolyticus</i>, <i>V. harveyi</i>, and <i>V. ordalii</i> DNA	Hirano <i>et al.</i> , 1998

Figure 6.1 An agarose gel of *Sau3A* I partial digests (I)

Genomic DNA of *V. carchariae* was partially digested with variable amounts of *Sau3A*I for 30 min at 37°C. After the reaction was terminated with addition of 1 µl of 0.5 M EDTA, the digested DNA fragments were electrophoresed on an 0.4 % agarose gel. A current of 50V was applied for the first 90 minutes, then 100V for 60 minutes.

Lane	Sample
1	1 kb ladder DNA marker
2	genomic DNA digested with 1000 mU <i>Sau3A</i> I
3	genomic DNA digested with 100 mU <i>Sau3A</i> I
4	genomic DNA digested with 50 mU <i>Sau3A</i> I
5	genomic DNA digested with 25 mU <i>Sau3A</i> I
6	genomic DNA digested with 15 mU <i>Sau3A</i> I
7	genomic DNA digested with 12.5 mU <i>Sau3A</i> I
8	genomic DNA digested with 10 mU <i>Sau3A</i> I
9	genomic DNA digested with 8.5 mU <i>Sau3A</i> I
10	genomic DNA digested with 5.0 mU <i>Sau3A</i> I
11	genomic DNA digested with 3.5 mU <i>Sau3A</i> I

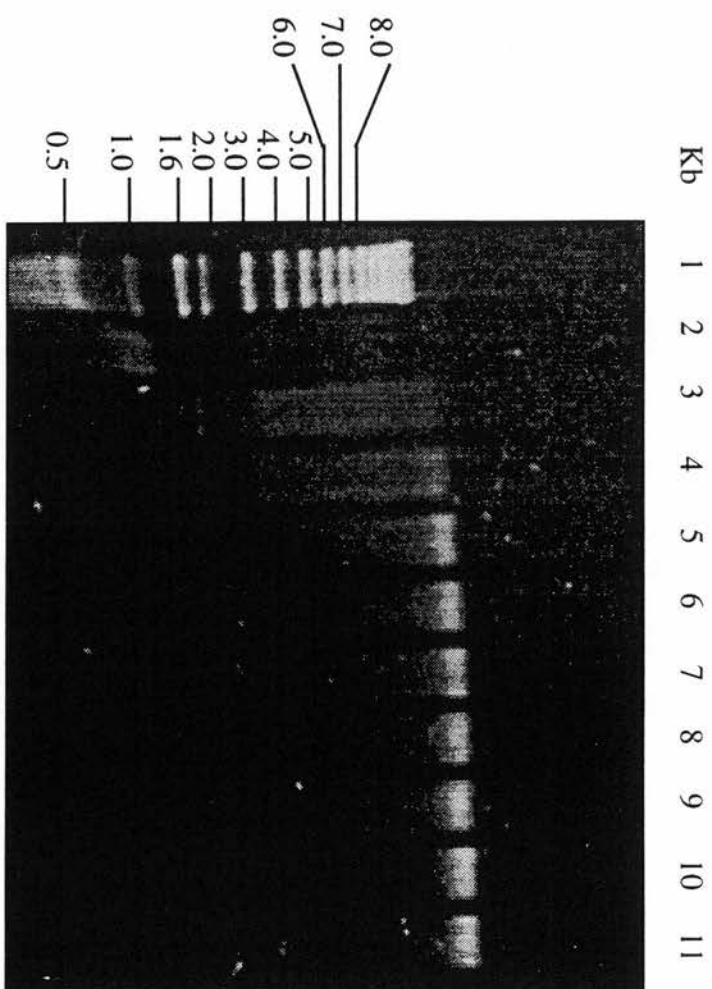
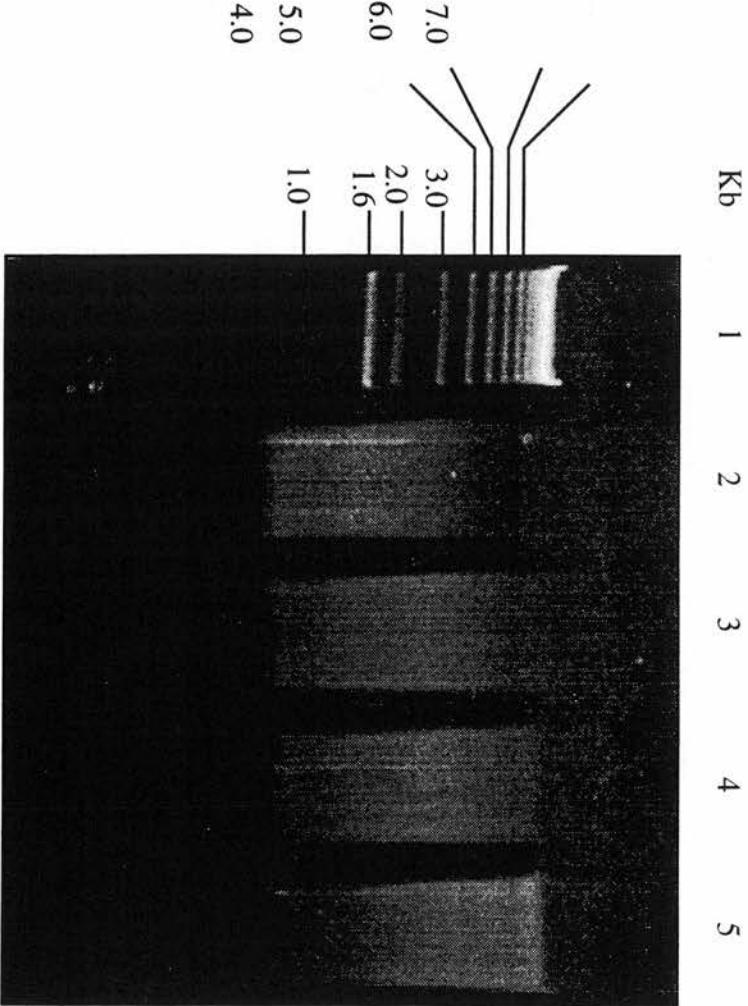


Figure 6.2 An agarose gel of *Sau3A* I partial digests (II)

Genomic DNA of *V. carchariae* was partially digested with a narrower range of *Sau3A*I dilution for 30 min at 37°C. After the reaction was terminated by addition of 1 µl of 0.5 M EDTA, digested DNA fragments were electrophoresed on a 0.4% agarose gel. A current of 50 V was applied for the first 90 minutes, then 100V for 60 minutes.

Lane	Sample
1	1 kb ladder DNA marker
2	genomic DNA digested with 500 mU <i>Sau3A</i> I
3	genomic DNA digested with 250 mU <i>Sau3A</i> I
4	genomic DNA digested with 167 mU <i>Sau3A</i> I
5	genomic DNA digested with 125 mU <i>Sau3A</i> I



reaction. In the large-scale preparation, 100 µg of genomic DNA was used and DNA concentration, time and temperature in the reaction were maintained identical to those used in the small-scale reactions.

6.2.2 Purification of *Sau*3A I Partial Digests on Agarose Gel

Partially digested DNA was further size-fractionated by preparative agarose gel electrophoresis using 1.5 % low melting temperature agarose. After electrophoresis, DNA fragments from 4 -7 kb were cut out of the gel (Figure 6.3A and 6.3B) and purified by the GELase method, followed by column chromatography using a High Pure PCR Product Purification Kit. After the purified DNA fragments were redissolved in 200 µl distilled water, the final concentration of the DNA solution was found to be approximately 5 ng/µl.

6.2.3 Ligation and Introduction of *Sau*3A I Partial Digests into *E. coli*

The pBluescript II KS(-) vector was used as a cloning vector. The cloning strategy was to ligate the purified DNA fragments into *Bam*H I sites of the prepared vector. After ligation reactions, salt in the reaction solution was removed by ethanol precipitation, and the recombinant plasmids were electroporated into competent *E. coli* cells strain XL1 Blue. The success of ligation was considered from the number of white colonies grown on LB agar plate containing ampicillin, IPTG and X-Gal and from double digestion analysis of miniprep DNA.

To obtain successful ligation, conditions were optimised by varying the molar ratio of DNA inserts to DNA vector. First of all, three different ratios of 1:3, 1:1, and 3:1 were tested. Dephosphorylating the ends of the *Bam*H I cut vector arms was used according to the suggestion in the standard Promega protocol. However, ligation was found to be unsuccessful by the conditions tested as indicated from very few white colonies detected. A broader range of the molar ratio (1:10, 1:5, 1:3, 1:1, 3:1, 5:1, and 10:1) was therefore tested. A comparison of the titres from various plates (Table 6.2) showed that lower yields of ligation (~12-13%) were obtained when lower molar

Figure 6.3 Large-scale preparation of *Sau3A* I partial digests

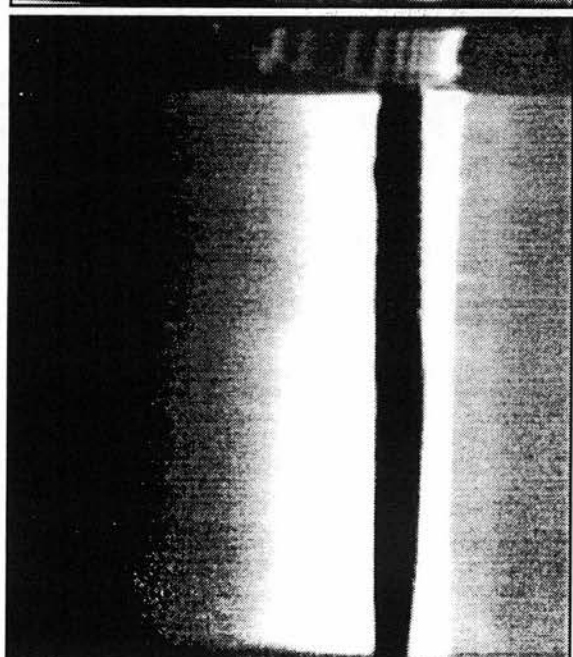
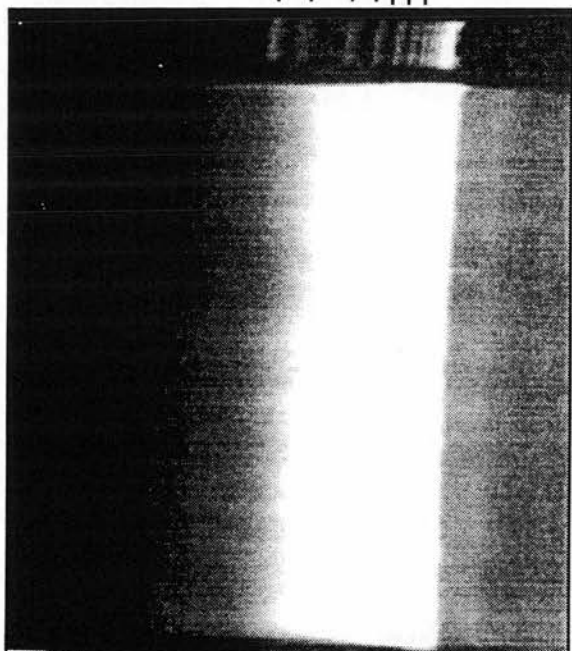
Genomic DNA of *V. carchariae* (100 µg) was partially digested with 250 mU *Sau3A* I. The digestion reaction was carried out in 4500 µl. The digestion conditions and concentration of DNA were maintained to be identical to those for the small-scale digestion. After the reaction was terminated, the DNA was collected by precipitation with ethanol and resuspended in 500 µl of TE buffer. The total volume of DNA solution was applied into a preparative well of a 1% low melting temperature agarose gel. Electrophoresis was performed at 4°C for 1 h and 30 min, 100 V. After electrophoresis, the DNA was viewed under UV light (Figure 6.3A), and a band corresponding to DNA size range of 4-7 kb was excised (Figure 6.3B), then purified by GELase method as described by the manufacturers. 1kb DNA ladder was used as a DNA marker.

A)

B)

Kb

7.0
6.0
5.0
4.0
3.0
2.0
1.6



4.7 Kb

Table 6.2 Optimisation of genomic DNA insert : pBluescript II KS (-) vector ligation.

Molar ratio (insert:vector)	Number of colonies observed			Transformation
	Blue	white	%	
No DNA control	1	0	0	
1:10	64	9	12	
1:5	35	5	13	
1:3	23	3	12	
1:1	21	8	28	
3:1	34	18	35	
5:1	37	26	41	
10:1	50	27	35	

ratios of DNA insert to DNA vector (1:10, 1:5, and 1:3) were used. On the contrary, higher molar ratios dramatically increased the ligation efficiency. The highest transformation efficiency (41%) was found at a molar ratio of 5:1. Consequently, this value was used for large-scale ligation. Prior to performing the large-scale ligation, it was confirmed that white colonies carried DNA inserts. Five single white colonies were picked randomly, then their plasmids were prepared by mini-scale preparation and double digested with *EcoR* I and *Xba* I. Figure 6.4 shows that DNA fragments of different sizes were generated as a result of enzyme digestion. The total size of the DNA bands was estimated from the gel and found to be larger than that of pBluescript vector, indicating that all the clones tested contained a vector carrying a DNA insert.

6.3 Genomic DNA Library Screening

Once the ligation was accomplished, the reaction was scaled up to obtain sufficient transformants required for representing the total number of the genes in genome of *V. carchariae*. The size of genomic library was worked out simply as follows:

$$\begin{aligned} \text{Size of genomic library} &= \frac{\text{insert size} \times \text{insert orientation}}{\text{size of } V. \text{ carchariae} \text{ genome}^*} \\ \text{(Number of white colonies required)} &= \frac{(4.7 \text{ kb}) \times 2}{4.2 \times 10^6} \end{aligned}$$

Average value between the smallest and the largest fragments (4kb and 7 kb) was considered.

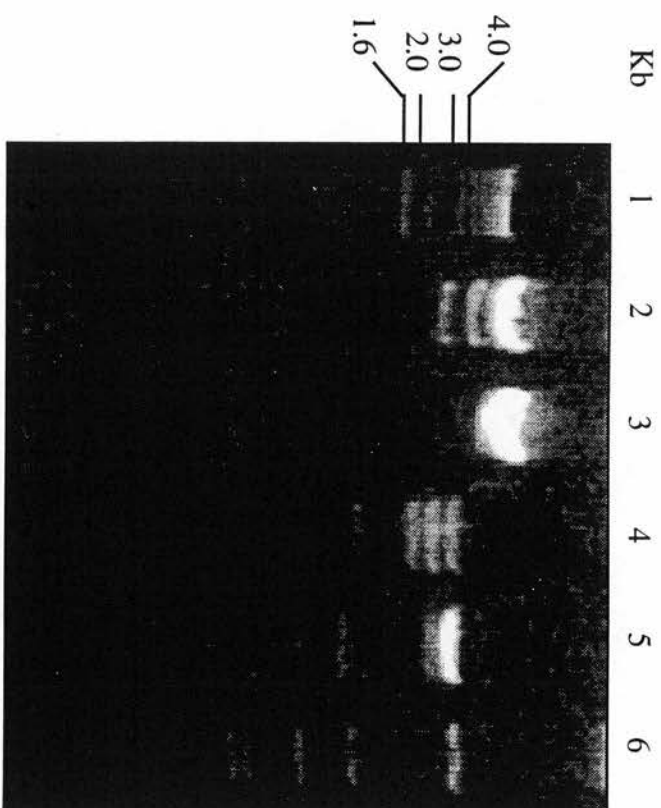
$$\begin{aligned} \text{So that the size of the library} &= \frac{[(4 \times 2) + (7 \times 2)] \times 10^3}{2 \times 4.2 \times 10^6} \\ &= 1,650 \text{ colonies} \end{aligned}$$

* The size of *V. carchariae* genome is assumed to be the same as of *E. coli* (4.2×10^6 bp) (Kaiser *et al.*, 1996).

Figure 6.4 Investigation of transformed *E. coli* for colonies carrying DNA inserts

Sau3A I partial digests were ligated to *BamH* I cut arms of pBluescript II KS(-) vector as given in section 2.2.19. and electroporated into competent *E. coli* cells strain XL1 Blue. The transformed cells were grown on an LB agar plate containing ampicillin, IPTG and X-Gal overnight at 37°C. Five single white colonies were picked and plasmid DNA was prepared by mini-scale preparation. Plasmid DNA was double digested with *EcoR* I and *Xba* I for 1 h at 37°C and analysed on an 1% (w/v) agarose gel.

Lane	Sample
1	1 kb ladder DNA marker
2-6	plasmids from five white colonies



To obtain sufficient transformed colonies for generating the genomic library in pBluescript vectors, four ligation reactions were carried out in this study. The library was screened for clones carrying chitinase using an immunological method. Expression of chitinase in clones that showed signals with anti-chitinase serum was further examined by the chitin plate assay.

6.3.1 Immunological Assay

Polyclonal antibodies raised against the purified *V. carchariae* chitinase were used to screen the genomic library. The colonies grown on LB containing ampicillin, IPTG and X-Gal were lifted onto a nitrocellulose membrane and identified by Western blot analysis as detailed in section 2.2.20.1. It was discovered that among approximately 2,100 transformants obtained, 12 colonies were found to give signal with anti-chitinase serum. To isolate single clones, a secondary screening was carried out. Areas that contained the positive colonies were isolated and grown in 3 ml LB/amp at 37°C with shaking for a few hours, then the cells were plated out with low density ($< 10^3$ cells per a 90 mm round plate). From twelve colonies obtained from the primary screening, eight single colonies designated P1C1, P1C2, P2C1, P2C2, P2C3, P3C1, P3C2, and P4C1 (P stands for plate and C stands for colony), were isolated in the secondary screening (Figure 6.5).

Confirmation of the expression of the chitinase gene was tested with all the positive clones (except P4C1) by Western blot analysis of proteins in cell extracts. Clone P4C1 was not tested for expression, but it was tested later because its single colony was not possible to isolate at this stage. Figures 6.6A and 6.6B shows that all the clones tested expressed high levels of a high molecular mass recombinant protein (~95 kDa) that reacted with anti-chitinase serum when the cells were grown in LB/amp containing 1% (w/v) swollen chitin. In addition, the expression level of the recombinant protein was found to be higher inside the cells than in the culture medium (Figures 6.6A and 6.7A). No chitinase was detected at all in the supernatant of all the clones when the cells were grown without chitin (Figure 6.7B). Some

Figure 6.5 Immunological screening of genomic library with anti-chitinase serum

Approximately 2100 white colonies obtained from four ligation reactions were screened for clones carrying a chitinase gene. All the colonies grown in LB agar plates containing ampicillin, IPTG and X-Gal were lifted onto a nitrocellulose membranes. After the cells were hydrolysed and fixed at 85°C for 30 min with 5% (w/v) SDS, Western blot analysed was performed as described for SDS-PAGE. Secondary screening was done with twelve areas that showed signals of antibody-antigen complexes with anti-chitinase serum raised against purified *V. carchariae* chitinase (see section 2.2.20.1 for detailed method).

Number	1	secondary screening, P1C1
	2	secondary screening, P1C2
	3	secondary screening, P2C1
	4	secondary screening, P2C2
	5	secondary screening, P2C3
	6	secondary screening, P2C4
	7	secondary screening, P3C1
	8	secondary screening, P3C2
	9	secondary screening, P3C3
	10	secondary screening, P4C1
	11	secondary screening, P4C2
	12	secondary screening, P4C3

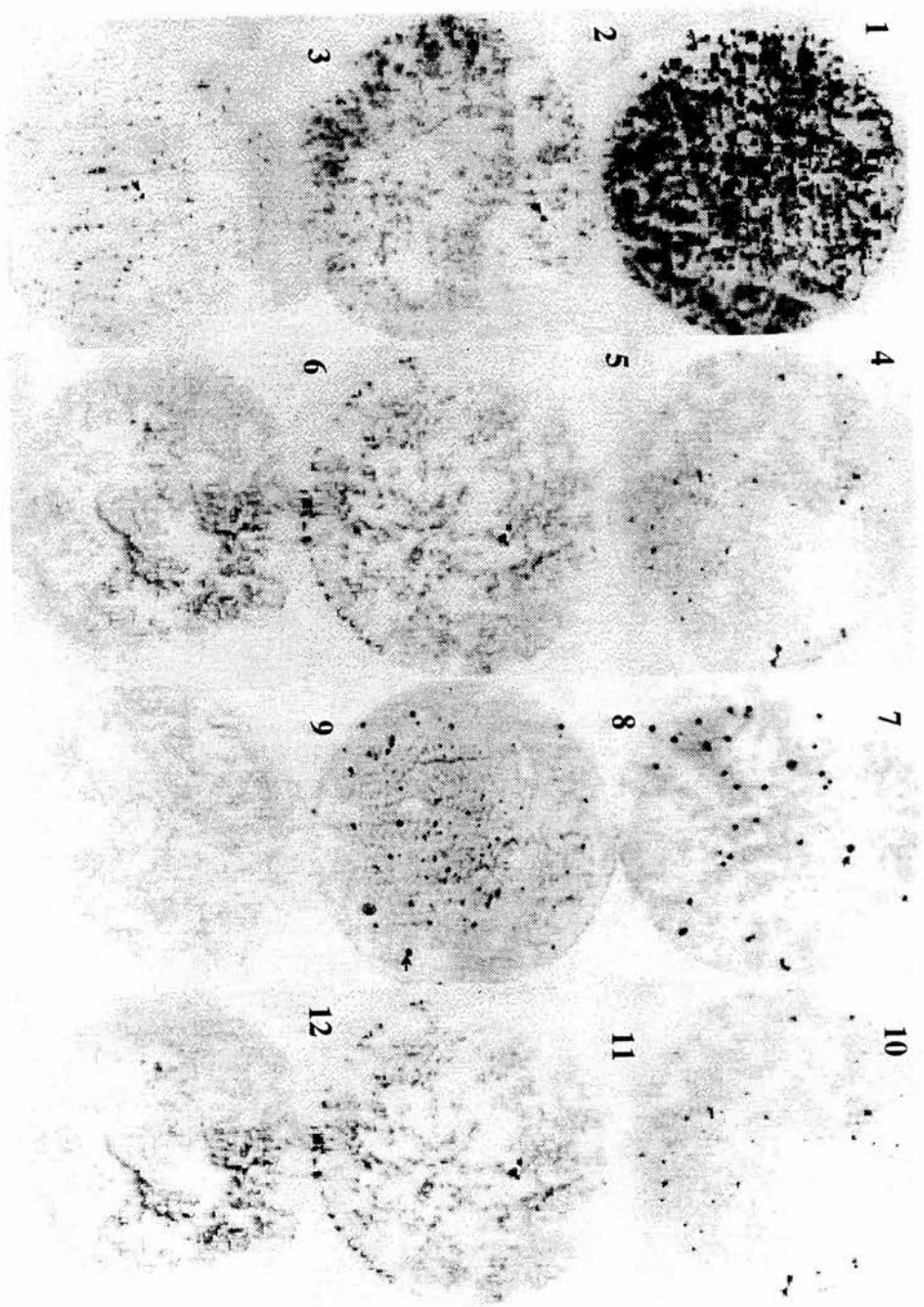
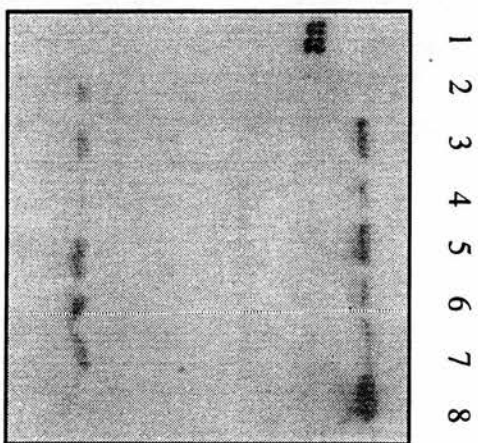


Figure 6.6 Western blot analysis of clones carrying DNA insert (I)

Single colonies of clones that gave signals with anti-chitinase serum were picked and grown overnight in LB media containing ampicillin at 37°C with 200 rpm shaking. Cell extracts of each clone were prepared as described in section 2.2.21 and analysed by Western blotting. Western blot analysis of the cell extracts when the cells were grown in the media supplemented with 1% (w/v) swollen chitin is shown in Figure 6.6.A and without chitin supplement is shown in Figure 6.6B

Lane	Sample
1	purified <i>V. carchariae</i> chitinase, 10 µg
2	cell extract of untransformed <i>E. coli</i> XL1 Blue
3	cell extract of P1C1
4	cell extract of P1C2
5	cell extract of P2C1
6	cell extract of P2C2
7	cell extract of P2C3
8	cell extract of P3C1
9	cell extract of P3C2

A)



B)

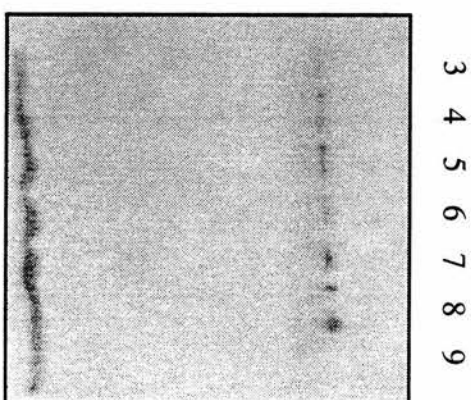
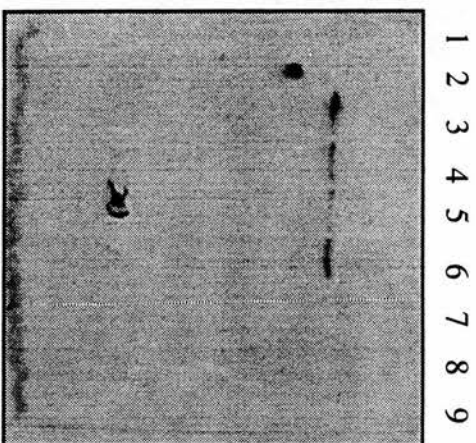


Figure 6.7 Western blot analysis of clones carrying DNA insert (II)

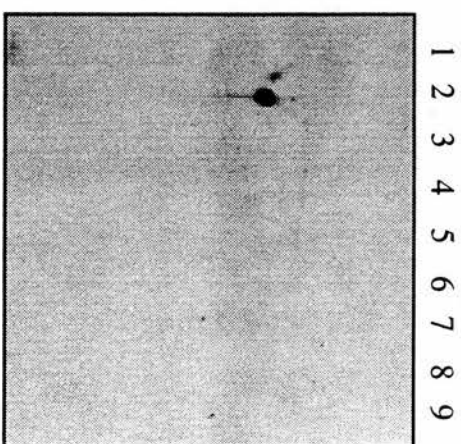
Single colonies of clones that gave signals with anti chitinase serum were picked and grown overnight in LB media containing ampicillin at 37°C with 200 rpm shaking. Concentrated supernatant samples of each clone (25 µl) were prepared from 3 ml of original growth media. The growth media were dialysed to remove salt before placing in on to the Speedvac concentrator. Western blot analysis of the supernatants when the cells were grown in the media supplemented with 1% (w/v) swollen chitin is shown in Figure 6.7.A and without chitin supplement is shown in Figure 6.7B

Lane	Sample
1	concentrated supernatant of untransformed <i>E. coli</i> XL1 Blue
2	purified <i>V. carchariae</i> chitinase, 10 µg
3	concentrated supernatant of P1C1
4	concentrated supernatant of P1C2
5	concentrated supernatant of P2C1
6	concentrated supernatant of P2C2
7	concentrated supernatant of P2C3
8	concentrated supernatant of P3C1
9	concentrated supernatant of P3C2

A)



B)



materials at the bottom of the gel (indicated by arrows) corresponded to the dye front, and were thought to be some non-specific binding.

6.3.2 Chitin Plate Assay

Expression of chitinase activity of all the positive clones was tested on an LB/amp agar plate containing 1% (w/w) swollen chitin. After one week of incubation at 30°C, degradation of chitin by the action of the chitinase was indicated by the clear zone produced around the stabbed site of each colony. The chitin agar plate result showed that different levels of chitinase activity were secreted from different clones. No chitinase activity was detected from P1C1 and P3C2, while P1C2, P2C1, P2C2, P2C3, and P3C2, secreted slight chitinase activity. The highest level of the enzyme activity was detected in clone P3C1 (Figure 6.8). The white regions in the plate corresponded to bacterial colonies.

6.4 Restriction Analysis of the Clones Expressing Chitinase Protein

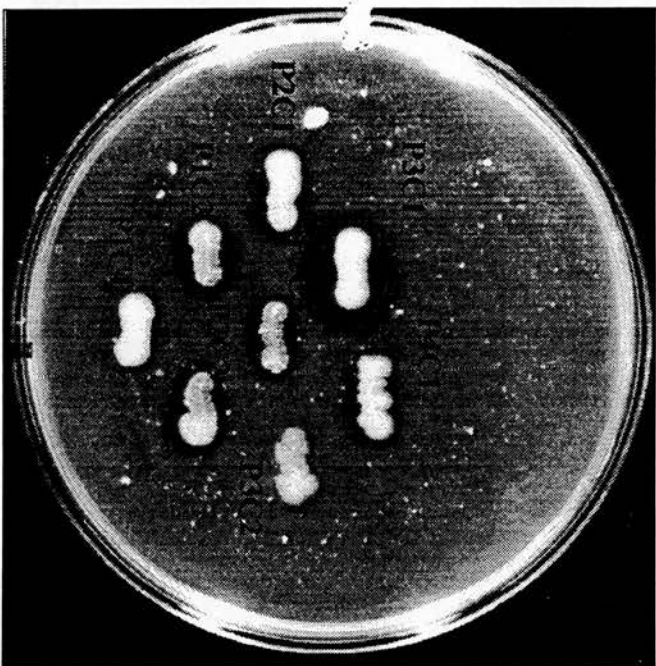
To identify clones producing recombinant chitinase, plasmid DNA samples from the positive clones (except P4C1) were prepared and digested with a single restriction enzyme (*EcoR* I), then their digestion patterns were analysed on agarose gel.

6.4.1 *EcoR* I Digestion

Figure 6.9 shows the digestion patterns of plasmid DNA from clones carrying an insert after being digested with *EcoR* I for 2 h at 30 °C. In all cases, three major DNA bands were found after digestion. Migration of the digested products in agarose gel were judged to be the same (approx. 5 kb, 2.5 kb, and 0.9 kb) except P3C1, in which three major DNA bands (approximately 6 kb, 2.5 kb and 0.8 kb) were observed. It should be mentioned that all those clones are identical at DNA level but it was found that they expressed differently on the chitin plate (see section 6.3.2). We did not understand at the time why they showed different chitinase activity but it has been

Figure 6.8 Chitin plate assay of clones carrying chitinase

Single colonies of clones that gave signals with anti-chitinase serum were picked and grown overnight in LB media containing ampicillin, then streaked to an LB agar plate containing ampicillin and 1% (w/v) swollen chitin. Cells were incubated at 30°C for one week, followed by another week at room temperature. Levels of chitinase expression are indicated by a number of plus (+) symbols; no expression is also indicated by minus (-).

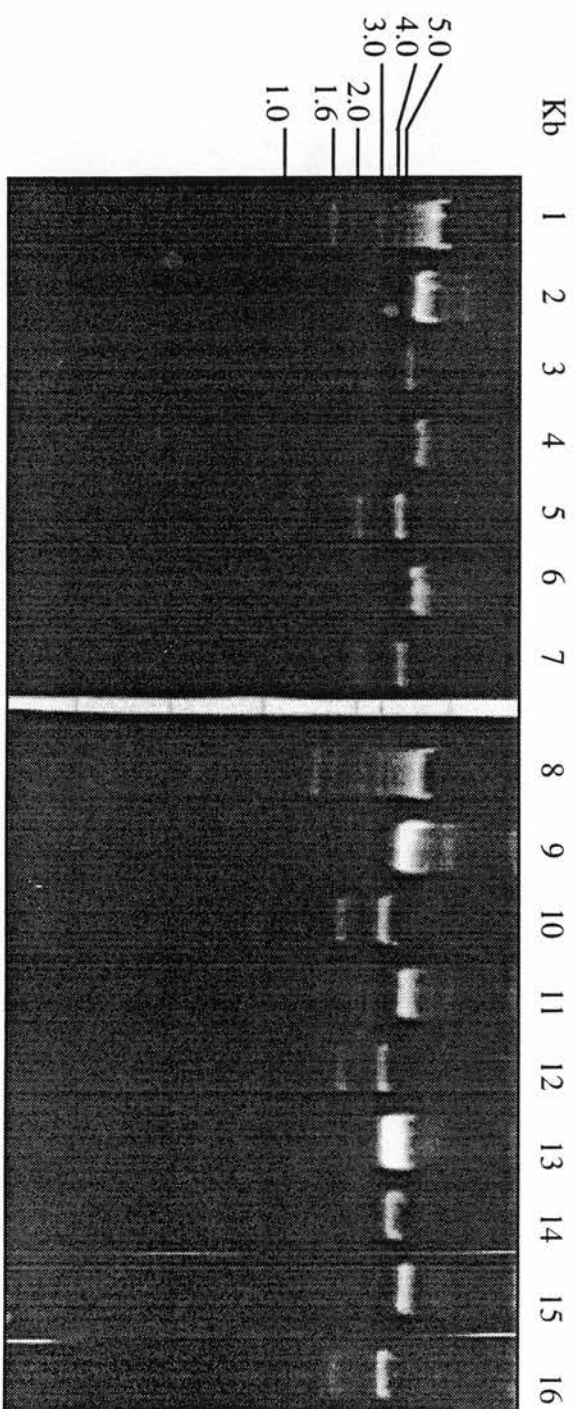


P1C1	-
P1C2	++
P2C1	+
P2C2	++
P2C3	++
P3C1	++++
P3C2	-
P4C1	++

Figure 6.9 *EcoR* I digestion of clone P1C1 and P3C1

Plasmid DNA of chitinase producing clones was prepared by small-scale preparation, and digested with *EcoR* I as detailed in sections 2.2.16.1 and 2.2.22.1.

Lane	Sample
1, 8	1 kb ladder DNA marker
2	clone P1C1, undigested
3	clone P1C1, <i>EcoR</i> I digested
4	clone P1C2, undigested
5	clone P1C2, <i>EcoR</i> I digested
6	clone P2C1, undigested
7	clone P2C1, <i>EcoR</i> I digested
9	clone P2C2, undigested
10	clone P2C2, <i>EcoR</i> I digested
11	clone P2C3, undigested
12	clone P2C3, <i>EcoR</i> I digested
13	clone P3C1, undigested
14	clone P3C1, <i>EcoR</i> I digested
15	clone P3C2, undigested
16	clone P3C2, <i>EcoR</i> I digested



shown later that most of the protein was expressed and accumulated in inclusion bodies, and only a very small amount of the protein was secreted.

6.4.2 Restriction Mappings of P1C1 and P3C1

Due to similarities in the *EcoR* I digestion patterns (except P3C1 that was slightly different from others), the clones were assumed to be identical. Clone P1C1 was chosen as a representative of all the apparently identical clones for further study because it showed no chitinase activity on the chitin plate. So, we expected that it might provide some explanation why different level of the enzyme activity was detected on the chitin plate. The restriction mapping of P1C1 was investigated in a comparison with clone P3C1. Ten restriction enzymes including *Kpn* I, *Sal* I, *Cla* I, *Pst* I, *Spe* I, *Xba* I, *EcoR* I, *Hind* III, *Sau3A* I, and *Xho* I, were used for this purpose. Figure 6.10A represents DNA patterns of P1C1 after digestion and Figure 6.10B represents DNA patterns of P3C1 after digestion. Figure 6.11A illustrates the restriction map of P1C1 and Figure 6.11B illustrates restriction map of P3C1. Similar patterns of restriction digestion were observed for both clones, although there were slight differences in the sizes of DNA fragments generated by each enzyme. It was shown that P1C1 carried a 5.5 kb DNA insert, whereas P3C1 carried a 4.0 kb DNA insert. The results also showed that both inserts contained no cleavage site for *Kpn* I, *Spe* I, *Xba* I, or *Xho* I. Moreover, P1C1 contained a unique site for *Sal* I, and *Cla* I and two cleavage sites for *Pst* I, *EcoR* I and *Hind* III. Similar results were found with clone P3C1 but it lacked a site for *Hind* III.

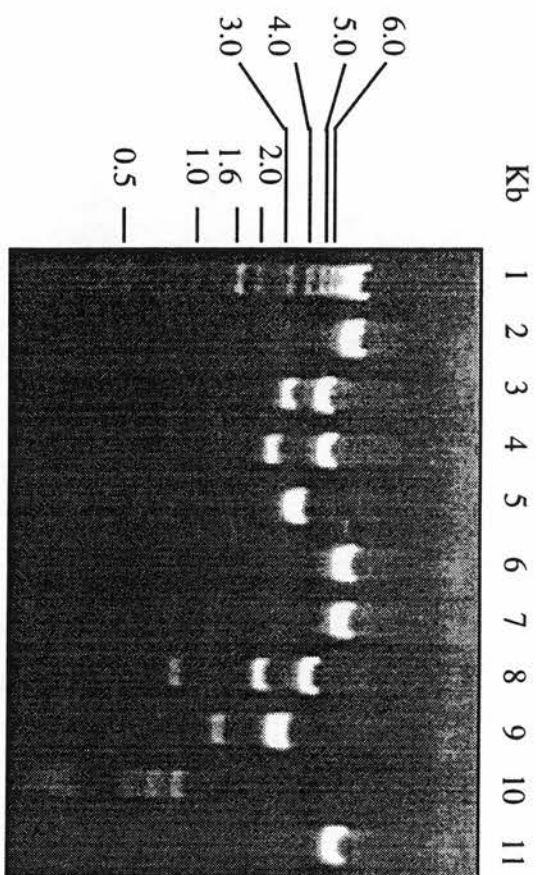
In addition, it was shown from Figures 6.11A and 6.11B that some part of the downstream end of the 4.0 kb fragment of P3C1 is extra, while some part of the upstream end is missing compared with the 5.5 kb fragment of P1C1. However, it was discovered later that both fragments contain the complete chitinase gene.

Figure 6.10 Restriction patterns of P1C1 and P3C1 on an agarose gel

Plasmid DNA of P1C1 and P3C1 was prepared by small-scale preparation, and digested with ten different restriction enzymes as detailed in sections 2.2.16.1 and 2.2.22.2. The restriction pattern of P1C1 is shown in Figure 6.10A and the restriction pattern of P3C1 is shown in Figure 6.10B.

Lane	Sample
1	1 kb ladder DNA marker
2	plasmid DNA digested with <i>Kpn</i> I
3	plasmid DNA digested with <i>Sal</i> I
4	plasmid DNA digested with <i>Cla</i> I
5	plasmid DNA digested with <i>Pst</i> I
6	plasmid DNA digested with <i>Spe</i> I
7	plasmid DNA digested with <i>Xba</i> I
8	plasmid DNA digested with <i>EcoR</i> I
9	plasmid DNA digested with <i>Hind</i> III
10	plasmid DNA digested with <i>Sau3A</i> I
11	plasmid DNA digested with <i>Xho</i> I

A)



B)

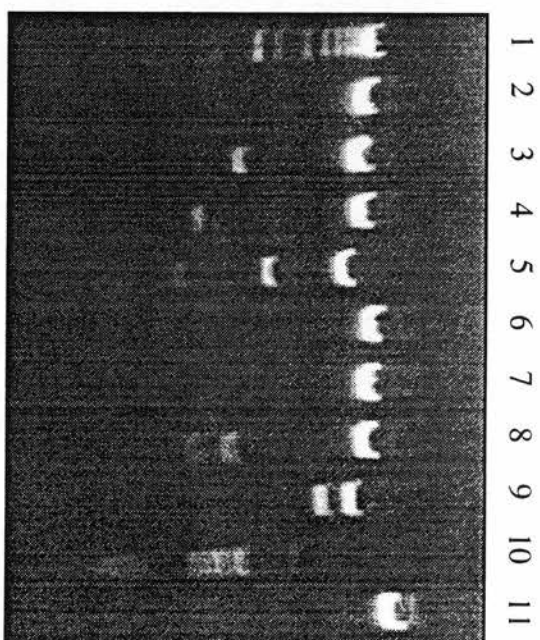
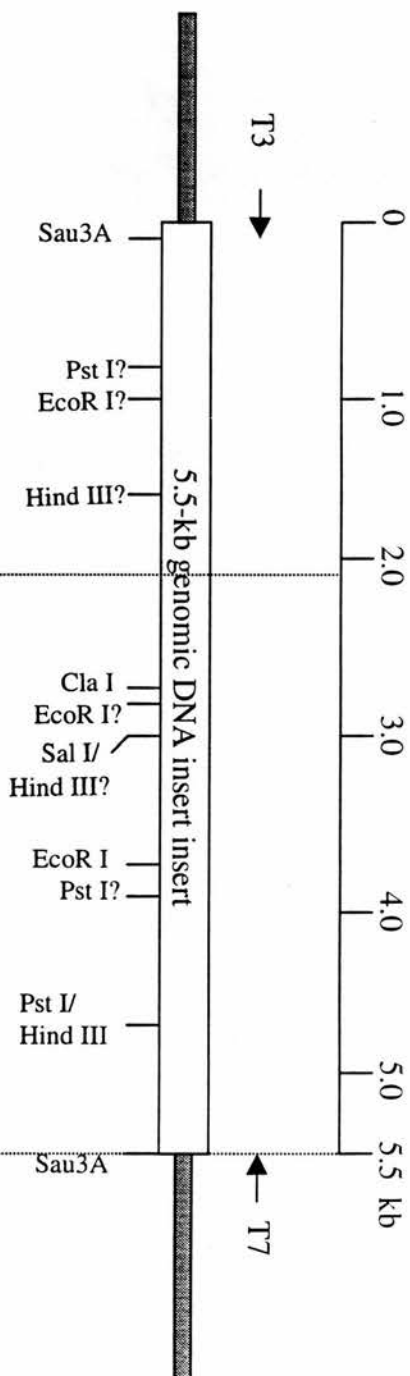


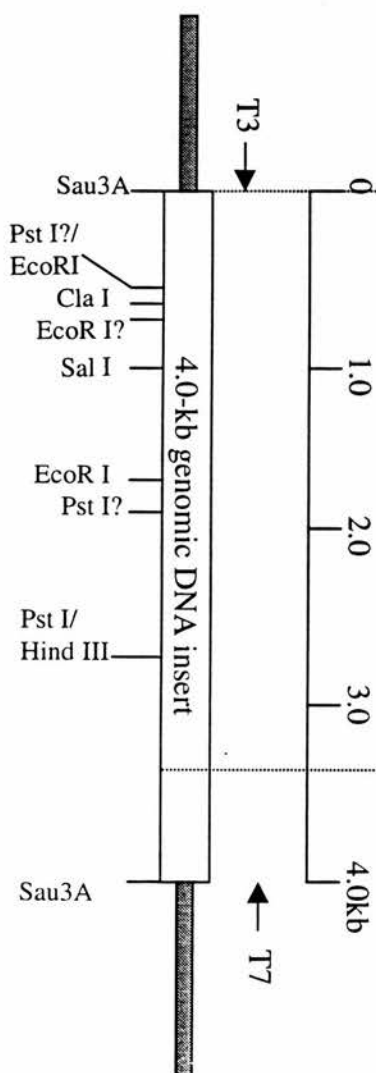
Figure 6.11 Restriction mappings of P1C1 and P3C1

A restriction map of P1C1 is illustrated in Figure 6.11A and a restriction map of P3C1 is illustrated in Figure 6.11B. Restriction sites with ambiguous order are indicated by question marks (?)

A)



B)



6.5 DNA Sequence Analysis of a Clone Expressing Chitinase Protein

6.5.1 Deletion Analysis of a Clone Carrying Chitinase

The purpose of this part of the study was to locate the gene encoding chitinase in the DNA insert so that a subclone carrying a smaller size of DNA insert with a complete coding sequence for the chitinase gene might be identified. Deletion analysis was done with P1C1 and P3C1 with three restriction enzymes, including *EcoR* I, *Sal* I, and *Cla* I, chosen based on the basis of restriction maps. Plasmid P1C1 was cut into two fragments with *Sal* I (~5.0 kb and 2.5 kb) and *Cla*I (~5.5 kb and 2 kb), but it was cut into three fragments with *EcoR* I (~5.0 kb, 1.5 kb and 1 kb). Plasmid P3C1 generated similar patterns with all three enzymes used, but their sizes were slightly different. DNA fragments (~6 kb and 1 kb), (~ 6.2 kb and 0.8 kb), and (~5.4 kb, 1 kb, and 0.6 kb) were generated with *Sal* I, *Cla* I and *EcoR* I, respectively (Figure 6.12).

The largest DNA bands from each digestion containing insert DNA were collected by agarose gel electrophoresis, and purified by QIAGEN plasmid purification kit. The purified DNAs were re-ligated and transformed into the same strain of *E. coli* as for the preparation of genomic library. Subclones of plasmid P1C1 were designated P1C1/*Sal* I, P1C1/*Cla* I, and P1C1/*EcoR* I, while subclones of plasmid P3C1, were designated P3C1/*Sal* I, P3C1/*Cla* I, P3C1/*EcoR* I. Expression of recombinant chitinase from all these subclones was checked by Western blotting.

Deletion analysis of plasmid P1C1 is illustrated in Figures 6.13A and 6.13B. Deletion analysis of plasmid P3C1 is illustrated in Figures 6.14A and 6.14B. Western blot analysis showed that strong signals with anti-chitinase serum were detected in subclones generated from *Cla* I (P1C1/*Cla* I and P3C1/*Cla* I). As shown in Figure 6.15, the antiserum recognised the protein bands produced by the subclones digested with *Cla* I at the same molecular weight as the undigested parental clones (~95 kDa). It was concluded at this stage that both clones might contain a complete chitinase gene in their insert. However, it was suggested from DNA sequence information that some part of the upstream end of the *Cla* I cut fragments

Figure 6.12 Deletion analysis of P1C1 and P3C1 (I)

Plasmid DNA samples from clones P1C1 and P3C1 were digested with three restriction enzymes (*EcoR* I, *Sal* I, and *EcoR* I) as detailed in section 2.2.23.1, then analysed on an agarose gel.

Lane	Sample
1	1 kb ladder DNA marker
2	clone P1C1 digested with <i>Sal</i> I
3	clone P1C1 digested with <i>Cla</i> I
4	clone P1C1 digested with <i>EcoR</i> I
5	clone P3C1 digested with <i>Sal</i> I
6	clone P3C1 digested with <i>Cla</i> I
7	clone P3C1 digested with <i>EcoR</i> I

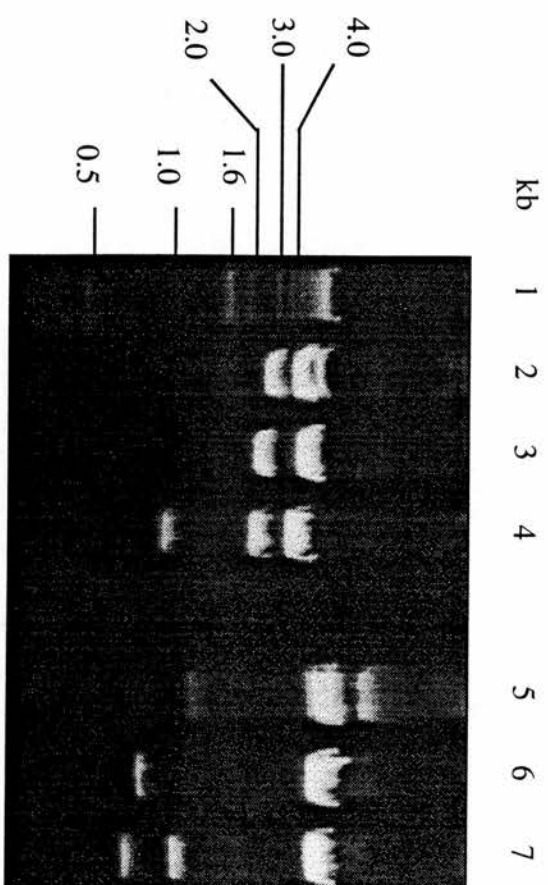
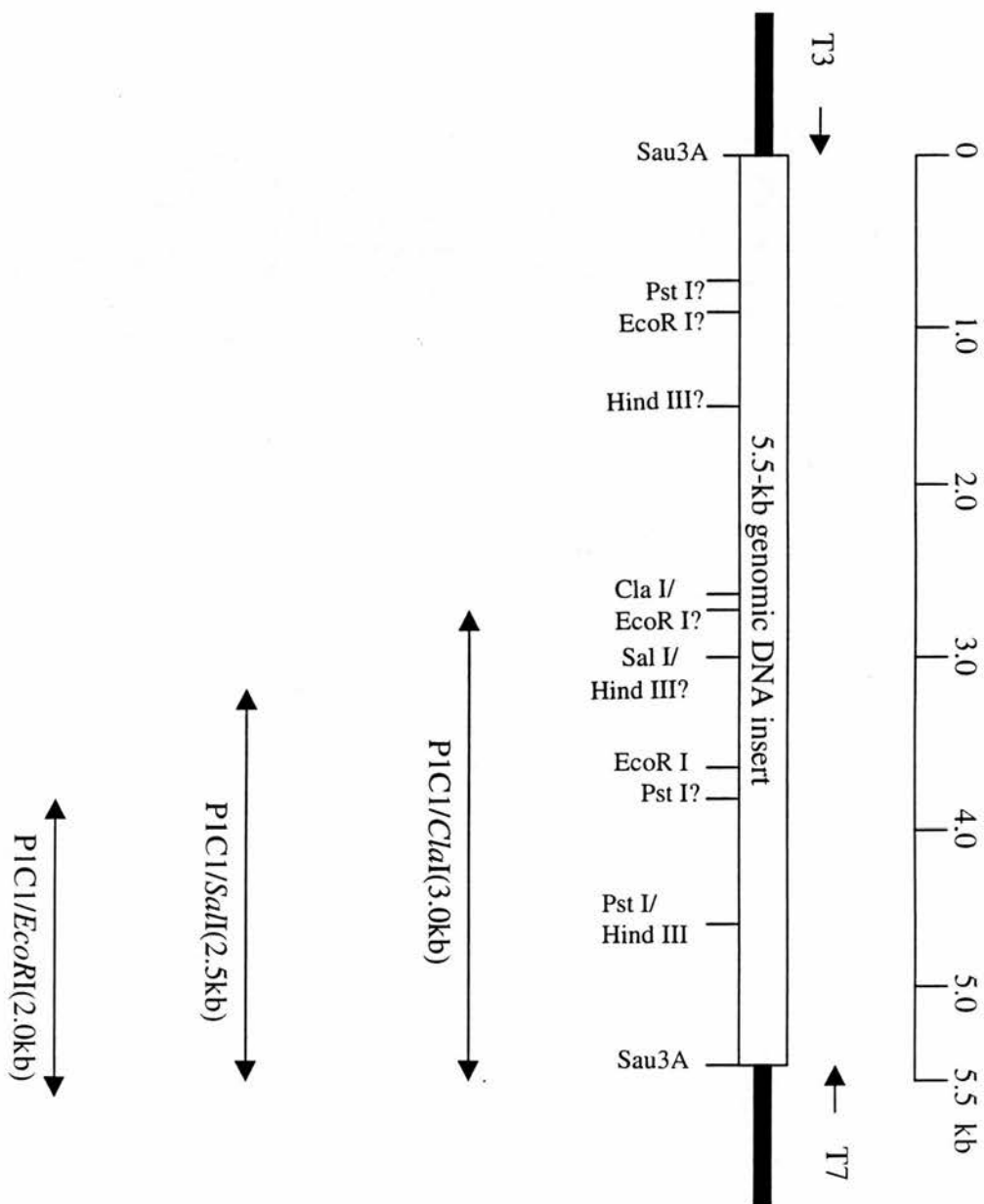


Figure 6.13 Deletion analysis of clone P1C1 (II)

Plasmid DNA of clone P1C1 was digested with three restriction enzymes (*Cla* I, *Sal* I, and *EcoR* I). After digestion, the largest DNA fragments viewed on a agarose gel were cut out of the gel and purified using a QIAGEN plasmid purification kit, and re-ligated before they were introduced into *E. coli* XL1 Blue by electroporation. Figure 6.13A represents deletion patterns of subclones P1C1/*Cla* I, P1C1/*Sal* I, and P1C1/*EcoR* I. Figure 6.13B represents Western blot analysis of chitinase expression from the subclones generated by the three enzymes.

A)



B)

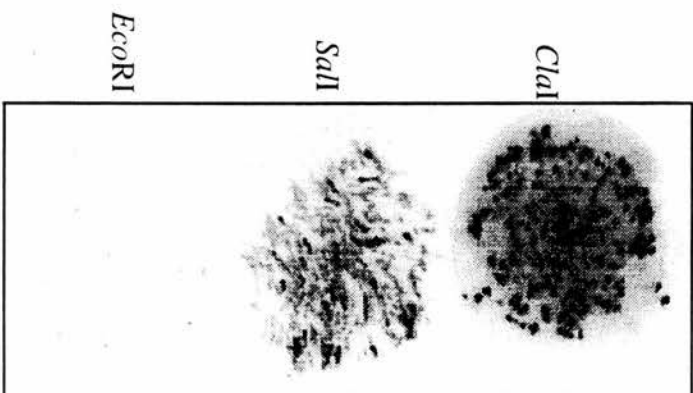


Figure 6.14 Deletion analysis of clone P3C1 (III)

Plasmid DNA of clone P3C1 was digested with three restriction enzymes (*Cla* I, *Sal* I, and *EcoR* I). After digestion, the largest DNA fragments viewed on a agarose gel were cut out of the gel and purified using a QIAGEN plasmid purification kit, and re-ligated before they were introduced into *E. coli* XL1 Blue by the electroporation method. Figure 6.14A represents deletion patterns of subclones P3C1/*Cla* I, P3C1/*Sal* I, and P3C1/*EcoR* I. Figure 6.14B represents Western blot analysis of chitinase expression from the subclones generated by the three enzymes.

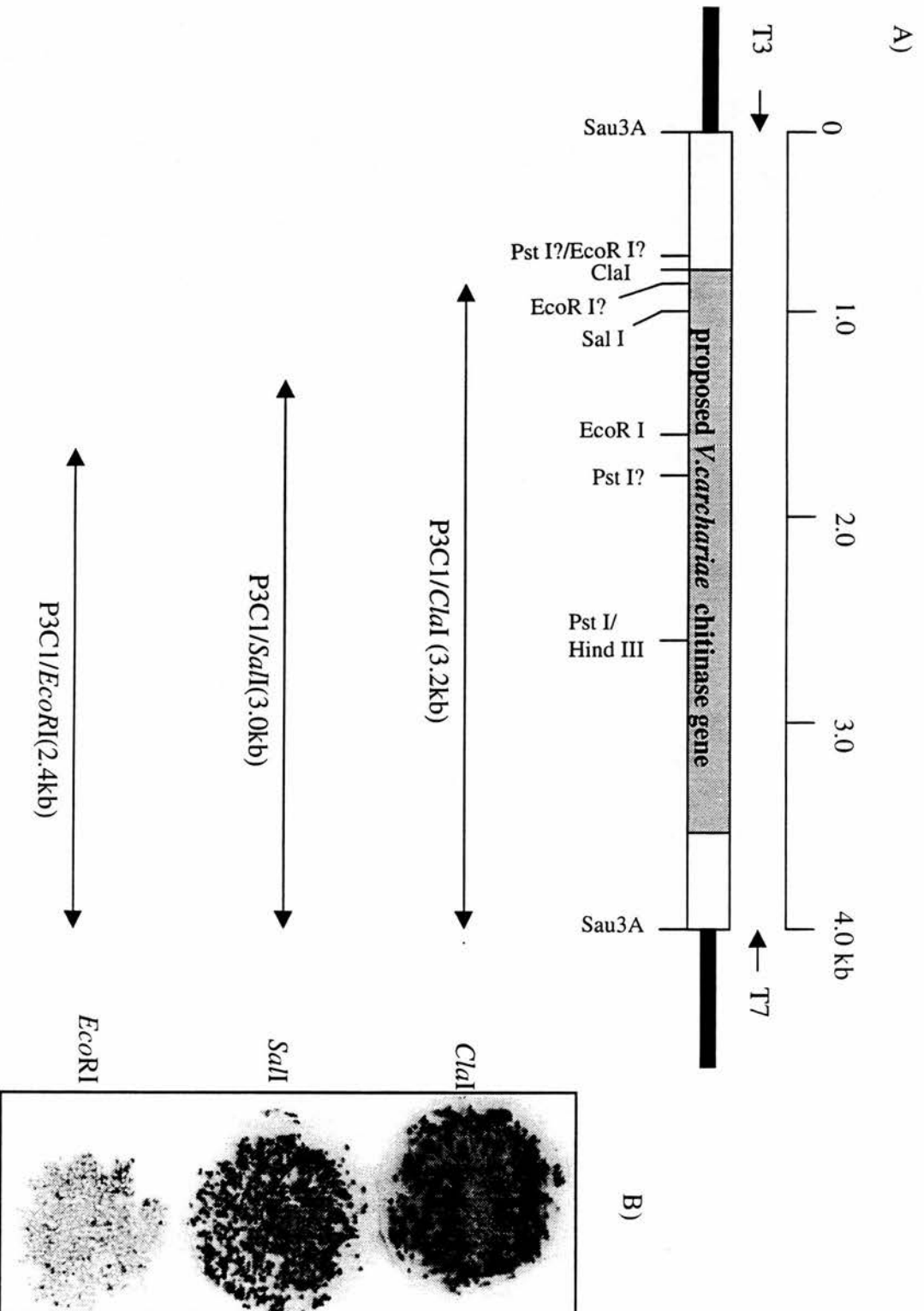
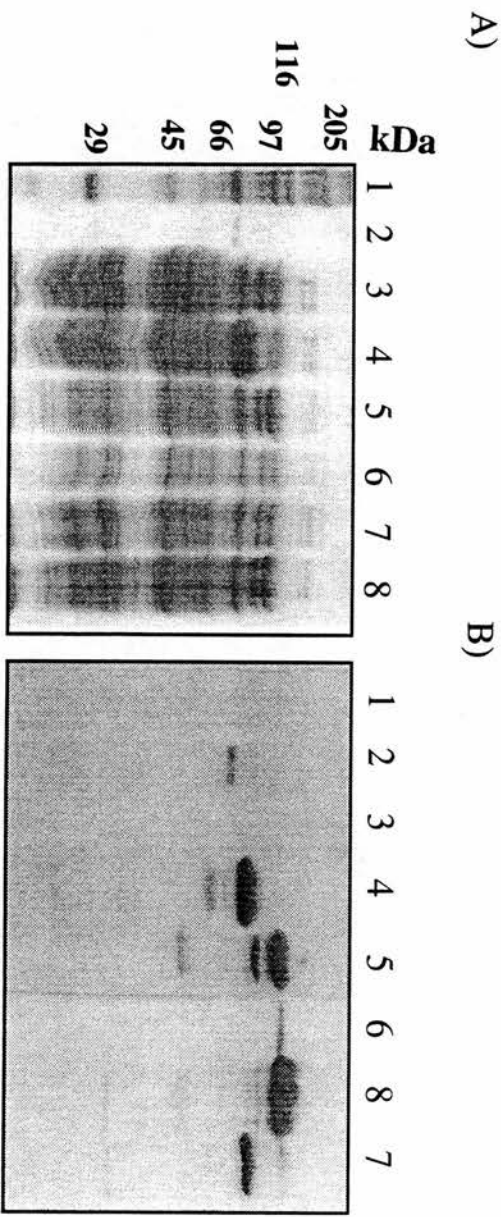


Figure 6.15 Chitinase expression of subclones P1C1/*Cla* I, P1C1/*Sal* I, P3C1/*Cla* I, and P3C1/*Sal* I

Single colonies of subclones P1C1/*Cla* I, P1C1/*Sal* I, P3C1/*Cla* I, and P3C1/*Sal* I were picked and grown overnight at 37°C in a 5-ml LB media containing ampicillin with shaking. One millilitre of cell culture was taken and centrifuged for 5 min. After the growth media were removed, the cell pellets were mixed with 150 µl of 3x SDS-sample buffer. After boiling for 5 min, samples (25 µl) were loaded onto two SDS-PAGE gels. After electrophoresis, one gel was Coomassie stained (Figure 6.15A) and the other gel was analysed by Western blotting (Figure 6.15B).

Lane	Sample
1	high molecular weight marker
2	purified <i>V. carchariae</i> chitinase (10 µg)
3	Cell extract of P1C1
4	Cell extract of P1C1/ <i>Sal</i> I
5	Cell extract of P1C1/ <i>Cla</i> I
6	Cell extract of P3C1
7	Cell extract of P3C1/ <i>Sal</i> I
8	Cell extract of P3C1/ <i>Cla</i> I



containing the chitinase gene lacked promoter control region. This implied that the gene without the control region was expressed by the promoter in the *lac* operon of the pBluescript vector instead of the native promoter. It was found that the incomplete chitinase gene was put in frame of the *Plac* promoter by loss of *Cla* I site in the DNA sequence (see Fig. 6.19), where the DNA fragment joined with the vector. In addition, this incidence was primarily noticed when the *Cla* I subclones were generated, it was not possible for the recombinant plasmids to be digested with *Cla* I enzyme.

Strong signals with the anti-chitinase serum were also detected in the cell extracts of the subclones digested with *Sal* I (P1C1/*Sal* I and P3C1/*Sal* I), but showed that the antiserum recognised a slightly lower molecular weight (~80 kDa) (see Figure 6.15) compared with the undigested clones. In addition, the protein seemed to correspond to the downstream part of the gene, indicating that some upstream part of the gene was removed when the DNA fragments were cut with *Sal* I. Explanation for chitinase expression was thought to be the same as for the subclones digested with *Cla* I. In the case for *Sal* I subclones, not only the DNA fragments carried the chitinase gene that lacked the control region, a few hundred basepairs at the upstream part of the chitinase gene were also missing. Therefore, the chitinase must have expressed under the *Plac* promoter in the vector. It showed no signal with anti-chitinase serum in cell extracts of the P1C1 subclone generated from *EcoR* I digestion (P1C1/*EcoR* I), and very faint signal with P3C1/*EcoR* I subclone. It appeared that these clones lacked the upstream part of the gene including the promoter control region and also part of the chitinase gene (~ 800-900 bp) (see Figure 6.14A for the proposed location of the chitinase gene). It might be that the remaining part of the gene joined the *EcoR* I site of the vector, but it was not put in frame with the *lacZ* gene of the vector so that the gene was not expressed. In other cases, the gene was put in frame but expressed weakly, or the protein was expressed at the same level as for the fragment from the *Cla* I and *Sal* I digestions, but the lacking part was important for antigenic recognition.

No chitinase activity was observed with any of the subclones when they were grown on chitin agar plates, even though the cells were incubated at 30°C for 2 weeks (data not shown). It was suggested from the structural study that the *N*-terminal region of the chitinase is involved in binding of substrate to the enzyme molecule (see chapter 7, section 7.7). This might be an explanation for the lack of chitinase activity detected in all the subclones that lacked the upstream part of the gene.

6.5.2 Nucleotide Sequence of subclone P3C1/*Cla*I

According to similarities in both the restriction patterns and the deletion analysis results, the clone P1C1 and the clone P3C1 appeared to contain the same single chitinase gene. However, plasmid P3C1 was considered to be more suitable for investigation of the entire sequence of the chitinase gene because of the smaller size of its DNA insert and the chitinase activity detected on the chitin agar plate as well as by standard assay (data not shown). It was concluded at this stage that subclone P3C1/*Cla* I might carry the entire sequence of the chitinase gene because it gave a strong signal with anti-chitinase serum at the same position as shown in the non-digested parental clone. It was therefore chosen for sequencing of the DNA insert. Prior to performing automatic sequencing of both nucleotide strands, about a hundred nucleotides at the upstream region of the P3C1/*Cla* I insert were checked for the existence of a chitinase coding sequence by manual sequencing using T3 as flanking primers (see Appendix V for the plasmid map of pBluescript II KS(-)). The partial sequence obtained was compared with all nucleotide sequences in the GenBank database. Searching results showed sequence similarity with ChiA from *Serratia marcescens*, and with ChiA from *Enterobacter* sp. A nucleotide alignment showed about 76% identity with that of ChiA from *Serratia marcescens* (Figure 6.16). The nucleotide region from 985 to 1086 of *S. marcescens* ChiA shows identity with the upstream part of P3C1/*Cla* I subclone.

Once the chitinase sequence identity was confirmed, automatic sequencing of both DNA strands was performed by Oswel according to a diagram illustrated in Figure 6.17. Even though both DNA strands were supposed to be sequenced, there are four

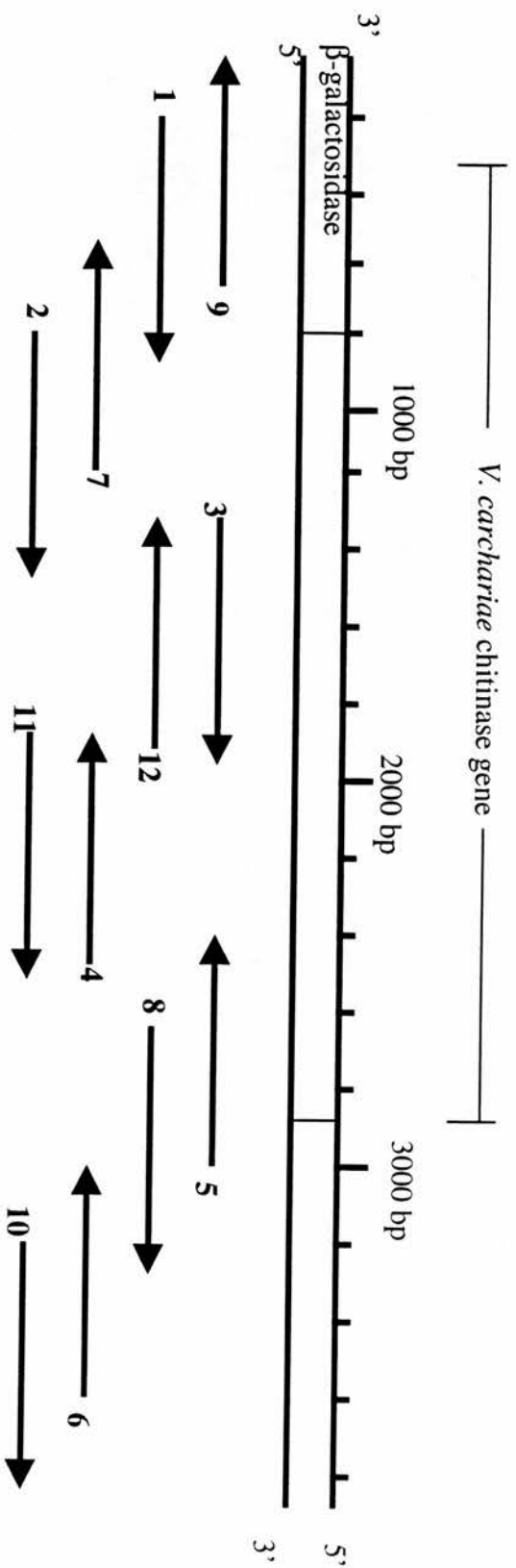
Figure 6.16 A nucleotide alignment of one hundred nucleotides at the upstream ends of subclone P3C1/*Cla* I (TEMPT3.SEQ) and the nucleotide sequence of ChiA from *Serratia marcescens* (SMCHIT.SEQ).

	↙990	↙1000	↙1010	↙1020	↙1030
SMCHIT.SEQ	ATCCCGGCGCAGAACCTGACCCACCTGCTGTACGGCTTTATCCCGATCTG				
TEMPT3.SEQ	ATGCCAGTTGATAACCTAACTCACATCCTTTACGGCTTTATCCCAATTG				
	↗10	↗20	↗30	↗40	↗50
	↙1040	↙1050	↙1060	↙1070	↙1080
SMCHIT.SEQ	CGGTGGCAACGGCATCAACGACAGCCTGAAAGAGATCGAAGGCAGCTTCC				
TEMPT3.SEQ	TGGT--CAAACGAATCAGTAAATCAGTTGGTGGTAACAGCTTAAATTGC				
	↗60	↗70	↗80	↗90	
SMCHIT.SEQ	AGG				
TEMPT3.SEQ	ACT				
	↗100				

Figure 6.17 Illustration of oligonucleotides designed for determination of the DNA sequence of subclone P3C1/*Cla*I

Positions where each nucleotide sequence was investigated by each oligonucleotide designed is indicated by arrows (→ for forward direction and ← for reverse direction). In principle, both nucleotide strands were sequenced, but there are four regions (bases 1-204, 1224-1285, 2544-2603, and 3671-3841) sequenced on only single strands.

Sequence No	Begin	End	Length
9	0	670	670
1	205	836	631
7	553	1223	670
2	731	1400	669
3	1286	1955	669
12	1297	1937	640
4	1873	2543	670
11	1886	2528	642
5	2448	3119	671
8	2604	3276	672
6	2999	3671	672
10	3179	3841	662



regions (bases 1-204, 1224-1285, 2544-2603, and 3671-3841) apparently found to be sequenced poorly (only single strand was determined). Two of these (bases 1224-1285 and 2544-2603) are in the chitinase gene. However, it was finally realised that the complementary nucleotides of these two regions were in fact determined, but they were not included in the diagram. These nucleotides were near the ends of the DNA fragments sequenced (fragment 12 for the region 1224-1285 and fragment 11 for the region 2544-2603), and were judged to be ambiguous nucleotides.

In total, 3841 nucleotides were sequenced, the sequenced was compared with all the sequences deposited in GenBank database. The sequence was converted into amino acids prior to making alignment. Sequences that showed high-scoring similarities with *V. carchariae* sequence are shown in Appendix VI. The searching result showed that the sequence had highest-scoring similarity with ChiA from *Alteromonas sp.*, followed by chitinase from *Enterobacter sp.*, and ChiA from *Serratia marcescens*. Characterisation of the detailed nucleotide sequence was based on the sequence of ChiA from *Alteromonas sp.*. Sequence analysis revealed a single open reading frame predicted to encode 848 amino acids which lacked some part of the upstream sequence including the promoter region, ribosome binding site, and the sequences encoding signal sequence and *N*-terminal amino acids. It appeared that the chitinase gene was expressed under control of the *Plac* promoter in pBluescript vector (Figure 6.18). This was confirmed by a dramatic increase in the amount of the protein band that reacted with anti-chitinase serum when the cells were grown in LB/amp with IPTG compared to when the cells were grown with 1% (w/w) swollen chitin (Figure 6.19).

6.5.3 A Complete Sequence of a Gene Encoding Chitinase

It was therefore clear that subclone P3C1/*Cla* I lacked some part of the native control region and the sequences encode a signal peptide and nine *N*-terminal amino acids. The original clone (P3C1) was used to investigate the upstream sequence of the chitinase gene that was missing in subclone P3C1/*Cla* I. Manual sequencing was

Figure 6.18 Nucleotide sequence of subclone of P3C1/*Cla* I

Nucleotide sequence of subclone P3C1/*Cla* I was investigated by automatic sequencing. The -10 and -35 regions of the β -galactosidase promoter sequence are highlighted. The putative ribosome-binding site (AGGA) is underlined. The amino acid sequence that belonged to the *V. carchariae* chitinase gene is in bold. The amino acid residues that belong to the β -galactosidase gene is not in bold.

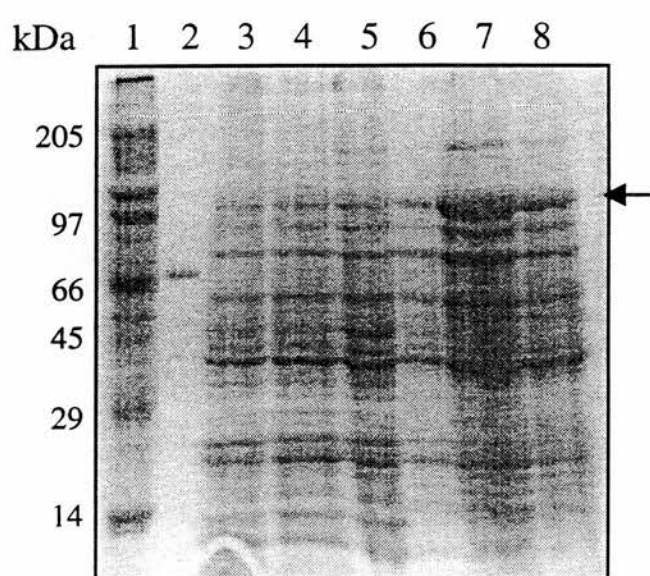
TATGTT

[illegible]

Figure 6.19 Effect of IPTG on chitinase expression of clone P3C1 and subclone P3C1/*Cla* I

Single colonies of clone P3C1 and subclone P3C1/*Cla* I were picked and grown overnight at 37°C in 5-ml LB media containing ampicillin and either 1%(w/v) swollen chitin or 0.5 mM IPTG. After that, cell extracts were loaded on a SDS-PAGE gel. After electrophoresis, the gel was Coomassie stained.

Lane	Sample
1	high molecular weight protein marker
2	purified <i>V. carchariae</i> chitinase (10 µg)
3	clone P3C1, no induction
4	clone P3C1, IPTG induction
5	clone P3C1, chitin induction
6	subclone P3C1/ <i>Cla</i> I, no induction
7	subclone P3C1/ <i>Cla</i> I, IPTG induction
8	subclone P3C1/ <i>Cla</i> I, chitin induction



performed using oligonucleotides designed near the 5'-end of the P3C1/*Cla* I sequence as DNA primers. The overlapping sequence obtained matched perfectly with the sequence of P3C1/*Cla* I so that purification of plasmid DNA (P3C1) was scaled-up and sent to Oswel for automatic sequencing using the same primers as for manual sequencing. After the sequence of the upstream part of the gene (approximately 600 nucleotides) was determined and the missing part of the gene discovered, it was assembled with the sequence of P3C1/*Cla* I and a complete sequence of the gene was obtained (Figure 6.20)

The nucleotide sequence that contained the complete chitinase gene of *V. carchariae* and its amino acid sequence were analysed. It revealed a predicted open reading frame consisting of 2550 bases starting at ATG codon and ending with the stop codon TAA, that coded for a predicted protein of 850 amino acids. The ATG initiation codon was preceded by a Shine-Dalgarno sequence (AGGA) that is typical of ribosome binding sites. Possible promoter sequences, TTGATT for the -35 region and TATGTT for the -10 region with 24 bp spacing between them, were proposed. An inverted repeat sequence (AAAAGGACAGC----GCTGTCCTTTT) that was composed of an 11-bp stem and a loop of four bases was found 44 nucleotides downstream from the termination codon of the open reading frame (see Figure 6.20). An *N*-terminal 21-amino acid signal sequence was found and consisted of a positively charged region and a hydrophobic region. Computer analysis revealed that 64% of the residues of the signal peptide are hydrophobic (Table 6.3) and is especially rich in alanine (36%) and leucine (14%) residues. A signal-sequence cleavage site was shown to be between alanine residue 21 and alanine residue 22 (von Heijne, 1983). This corresponded with the alanine that was found to be the first residue in the *N*-terminal amino acid sequence of the *V. carchariae* chitinase determined by protein sequencing (see chapter 4.6.3).

Some predicted structural properties of the whole chitinase and the proposed mature chitinase are given in Table 6.4. The proposed mature chitinase was obtained by removal of the signal peptide (the first 21 amino acid residues), and the amino acids after residues 593 from the whole protein. The C'-terminal end of the precursor

Figure 6.20 The entire nucleotide sequence of *V. carchariae* chitinase

The complete sequence of the chitinase gene was obtained by joining the upstream sequence (containing the chitinase control regions, transcriptional starting site, Shine-Dalgarno sequence, the sequence encoding the signal peptide and 9 *N*-terminal amino acids) determined from clone P3C1 with the nucleotide sequence encoding 819 amino acids determined from subclone P3C1/*Cla* I. The -10 and -35 regions of a possible promoter sequence are highlighted. The transcriptional start site is underlined. The putative ribosome-binding site (AGGA) is in bold with an underline. The signal sequence containing 21 amino acids is boxed. The proteolytic cleavage site is shown with a vertical arrow (↓). The *N*-terminal amino acid sequence of *V. carchariae* chitinase was determined by an Applied Biosystem 477A microsequencer and is underlined. The putative inverted-repeat sequence downstream of the determination codon (asterisks) is indicated by facing arrows with solid lines.

Table 6.3 Signal peptide fragment (1-22) composition analysis

Amino Acids	Number count	%by weight	%by frequency
Charged (RKH CYDE) 2		12	9
Acidic (DE)	0	0	0
Basic (KRH)	1	7.4	4.6
Polar (NCQSTY)	4	20	18
Hydrophobic (AILFWV)	14	60	64
A Ala	8	27.02	36.36
C Cys	1	4.90	4.55
D Asp	0	0.00	0.00
E Glu	0	0.00	0.00
F Phe	1	6.99	4.55
G Gly	2	5.43	9.09
H His	0	0.00	0.00
I Ile	1	5.38	4.55
K Lys	0	0.00	0.00
L Leu	3	16.13	13.64
M Met	1	6.23	4.55
N Asn	2	10.84	9.09
P Pro	0	0.00	0.00
Q Gln	0	0.00	0.00
R Arg	1	7.42	4.55
S Ser	1	4.14	4.55
T Thr	0	0.00	0.00
V Val	1	4.71	4.55
W Trp	0	0.00	0.00
Y Tyr	0	0.00	0.00

Table 6.4 Predicted structural class of the *V. carcharia* chitinase

Analysis	Whole Protein		Mature Protein	
			(Amino acid residues 22-593)	
Molecular weight	90,249		62,254	
Length	850		572	
1 microgram =	11.1 pMoles		16.1 pMoles	
Molar Extinction coefficient	152140±5%		16063±5%	
1A(280) =	0.59 mg/ml		0.55 mg/ml	
Isoelectric Point	4.33		4.36	

protein was removed based on the multiple alignment of four chitinases, ChiA from *Altermonas sp.*, ChiA from *S. marcescens*, chitinase from *Autographa californica* nuclear polyhedrosis virus, and chitinase from *Orgyia pseudotsugata* nuclear polyhedrosis virus (data not shown), that show sequence similarities with the precursor 95 kDa chitinase. The molecular weight of the whole protein is calculated to be 90,249, while the mature protein is calculated to be 62,254. The calculated mature protein was found to be similar to the native *V. carchariae* chitinase estimated on SDS-PAGE (63,000). The properties of the whole protein and the mature protein were generally similar to each other.

6.6 Expression of Recombinant Chitinase in *E. coli*

6.6.1 Localisation of Recombinant Chitinase in *E. coli*

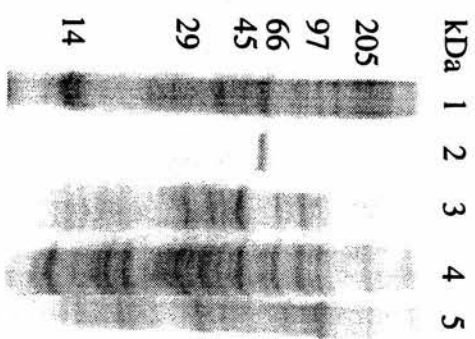
We expected the recombinant chitinase would be processed and secreted outside the cells, but it was found that the protein was neither processed nor secreted. It was shown in Figures 6.6 and 6.7 that the majority of the expressed recombinant chitinase was detected inside the cells. Different cell fractions including periplasmic and cytoplasmic fractions were therefore investigated for chitinase using Western blot analysis in order to indicate where the protein was stored in the cells. Even though the experiment was repeated a few times, it revealed that no signal of anti-chitinase serum-chitinase complexes was detected in the two fractions tested. However, a strong signal was observed with the precipitate that was removed from the cytoplasmic fraction after centrifugation. It was suspected that the protein might be produced and stored in inclusion bodies. This hypothesis was confirmed as shown in Figure 6.21, where a strong signal was detected in the inclusion body fraction. Moreover, no signal at all was detected in the periplasmic or cytoplasmic fractions. In addition to the 95 kDa band recognised by the antiserum, there were some smaller bands also detected. These were thought to be some degradation products or truncated proteins. Truncated products appear to occur when the translation process is working very fast. It has been shown, in both prokaryotes and eukaryotes, that

Figure 6.21 Localisation of the recombinant chitinase from *E. coli*

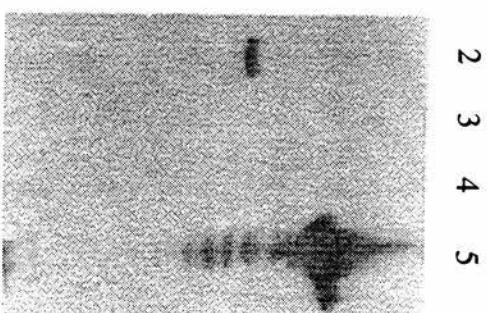
A single colony of clone P3C1 was picked and grown overnight at 37°C with 200 rpm shaking in LB medium containing ampicillin and 1% (w/v) swollen chitin. Different cellular fractions (periplasmic fraction, cytoplasmic fraction, and inclusion bodies) were prepared as described in section 2.2.24.1 and 2.2.24.2. Each fraction was loaded onto two SDS-PAGE gels. After electrophoresis, one gel was Coomassie stained (Figure 6.21A), and another gel was analysed by Western blotting (Figure 6.21B).

Lane	Sample
1	high molecular weight marker
2	purified <i>V. carchariae</i> chitinase (10 µg)
3	periplasmic fraction
4	cytoplasmic fraction
5	inclusion body fraction

A)



B)



some errors occur during translation process. The errors could be either results of insertion, frameshifting, tRNA hopping, or termination bypass (Manuel *et al.*, 1993).

6.6.2 Protein Processing Study

It was concluded from Western blot results that the recombinant chitinase was expressed as a precursor since a high molecular weight of the protein (~95 kDa) was observed throughout the expression study. An experiment was set up to determine how the processing process may occur in *E. coli*. The cell extract of clone P3C1 was incubated at 30 °C with the cell extract of *V. carchariae* for different times (1h, 2h, 4h and overnight), and possible changes in position of protein pattern was analysed by Western blotting. In this study, we were hoping that the recombinant protein could be processed when it was mixed with the cell extract of *V. carchariae* cell extract. However, results showed that no changes in protein pattern were observed either under denaturing conditions (Figure 6.22A) or non-denaturing conditions (Figure 6.22B) in the presence or absence of *V. carchariae* cell extract. In addition, it was found that the position and pattern of the signal observed in *V. carchariae* cell extract had no difference from the cell extract of *E. coli*, but were different from *V. carchariae* mature chitinase.

6.7 Conclusions and Discussion

6.7.1 Preparation of Genomic DNA Library from *V. carchariae*

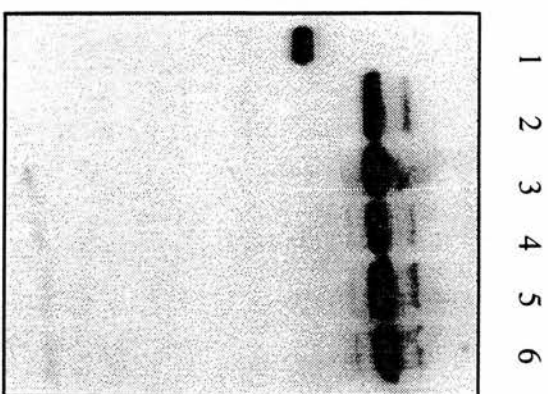
To isolate the gene encoding chitinase, a DNA library was prepared from genomic DNA of *V. carchariae* partially digested with *Sau3A* I. Because of their compatibility with the *Bam*H I cleavage site (GGATCC), the enzymes *Sau3A* I and *Mbo* I are commonly used in a genomic library preparation. Both enzymes recognise the sequence GATC, which occurs on average every 4⁴ (256) bp in random sequence DNA. The enzymes are therefore judged to cut sufficiently frequently so that a random library containing a set of overlapping fragments can be generated.

Figure 6.22 Protein processing of the recombinant chitinase in *E. coli*

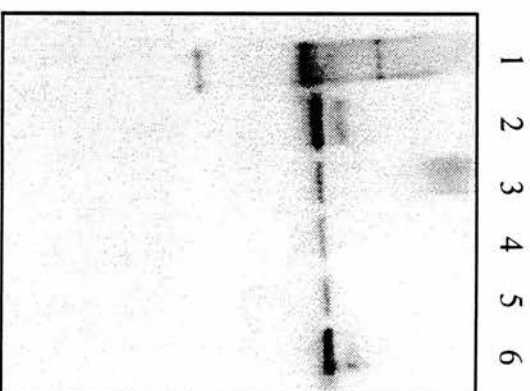
Cell extracts of clone P3C1 and of *V. carchariae* were freshly prepared as described in section 2.2.24.1. The cell extract of clone P3C1 (1.0 ml) was mixed with cell extract of *V. carchariae* (0.5ml). Each extract mixture was incubated separately at 37°C at different times of 1 h, 2 h, 4 h, and overnight. 20 µl of each extract mixture was loaded onto a SDS-PAGE gel as well as a non-denaturing PAGE gel. After electrophoresis, both gels were analysed by Western blotting. Figure 6.22A is Western blotting of SDS-PAGE and Figure 6.22B is Western blotting of non-denaturing PAGE.

Lane	Sample
1	purified <i>V. carchariae</i> chitinase, 10 µg
2	cell extract of <i>V. carchariae</i>
3	cell extract of P3C1 + cell extract of <i>V. carchariae</i> , 1 h
4	cell extract of P3C1 + cell extract of <i>V. carchariae</i> , 2 h
5	cell extract of P3C1 + cell extract of <i>V. carchariae</i> , 4 h
6	cell extract of P3C1 + cell extract of <i>V. carchariae</i> , overnight

A)



B)



The minimum size of a genomic library required for a complete range of 4-7 kb DNA fragments was initially simply estimated to contain about 1,650 transformants (see section 6.3). However, the above number was likely to be underestimated by a factor of 2.3 if the following formula that was suggested to be generally accepted (Kaiser *et al.*, 1996) was used to estimate the size of the library.

$$\text{Size of library (N)} = \frac{\ln(1-p)}{\ln(1-x/y)}$$

If x is the insert size (4-7 kb) and y is the size of the haploid bacterial genome (it was assumed that the haploid genome of *V. carchariae* is identical to that of *E. coli*). The probability (p) of containing any particular DNA sequences was set to be p=0.99. Consequently that the library size is estimated to be:

$$\begin{aligned} N &= \frac{\ln [1-0.99]}{\ln [1-(4 \times 10^3 / 4.2 \times 10^6)]} + \frac{\ln [1-0.99]}{\ln [1-(7 \times 10^3 / 4.2 \times 10^6)]} \\ &= \frac{2}{2} \\ &= 3,797 \text{ colonies} \end{aligned}$$

According to the library size above, the genomic library prepared (2100 colonies) in the actual experiment was not considered to represent a truly random genomic library and might not serve as a sufficient collection of DNA fragments of *V. carchariae* genome. However, the immunological screening result proved that this number of colonies was sufficient for the isolation of chitinase gene in this study.

In the preparation of the genomic library, successful ligation of DNA inserts to the pBluescript vector appeared to be a major problem that needed to be overcome at an early stage. Phosphorylation of the ends of *Bam*H I cut pBluescript vectors was used, as a strategy suggested by many standard protocols to help ligation efficiency by preventing self-ligation of DNA vector thereby reducing the background of non-recombinant colonies. This approach was tried unsuccessfully with ligation of *Sau*3A I partial digests here. It prevented self-ligation of non-recombinant colonies, but only

a few recombinant colonies (indicating unsuccessful ligation) were ever obtained. Successful ligation was never achieved until it was realised that a sufficient amount of DNA inserts in the ligation reaction and an appropriate molar ratio of DNA fragments to vector seemed to determine the success of ligation. However, the increase in ligation efficiency also appeared to be a result of the addition of *Bam*H I in the ligation reactions. This was because a linear structure of the DNA vector was always maintained by action of *Bam*H I in the reaction mixture. As a result, it allowed a higher possibility for *Sau*3A I cut arms of DNA inserts to encounter *Bam*H I cut arms of DNA vector and to ligate them together.

6.7.2 Isolation of Clones Carrying a Chitinase Gene

Immunological detection using anti-chitinase serum raised against the purified *V. carchariae* chitinase was found to be a very highly specific and sensitive screening method. The technique developed directly from the colonies grown on the selective LB agar plate allowed the experiment to proceed promptly and detection of chitinase producing clones was possible to achieve within a week.

Even though expression of chitinase of all positive clones isolated from LB agar plate was confirmed by SDS-PAGE and Western blotting, it was found that not all the clones had activity on the chitin substrate on chitin agar plates. Only six clones (P1C2, P2C1, P2C2, P2C3, P3C1, and P4C1) out of eight clones (not P1C1 and P3C2) expressed the 95 kDa chitinase and stored it inside the cells as well as secreted some active enzyme outside to the growth medium. This was indicated from the clear zone produced around its colony, whereas P1C1 and P3C2 expressed an apparent enzyme that was stored it inside the cells. Moreover, different levels of both active and inactive expressed chitinase were observed in the clone. This is proposed to be a result of protein folding and processing effects (see section 6.7.7 for detailed discussion). The absence of the enzyme activity could be because the bacterial cells did not secrete the chitinase at all but kept it inside the cells, or the cells secreted the protein but the protein was inactive.

6.7.3 Expression of the Chitinase Gene in *E. coli*

The chitinase gene in *E. coli* was found to be expressed under control of its own promoter rather than using a strong vector-carried promoter region. This was indicated from a remarkable increase in expression level of chitinase when the cells were grown in the LB/amp medium containing 1% (w/v) swollen chitin as shown in the Western blot in Figure 6.7A and 6.7B. However, an attempt to create clones that carried a smaller DNA insert for nucleotide sequence purposes revealed that expression of the subclones generated by *Cla* I and *Sal* I (P1C1/*Cla* I, P1C1/*Sal* I, P3C1/*Cla* I and P3C1/*Sal* I) was under the control of *Plac* promoter in the *Plac* gene of the pBluescript vector. This was confirmed by the consensus sequences at -35 region (TTACAC) and at -10 region (TATGTT) and ribosome binding site (AGGA) of the *Plac* promoter found in the nucleotide sequence of P3C1/*Cla* I. In addition, the sequence analysis showed that the sequence encoding chitinase was in the same open reading frame as β -galactosidase in the *lacZ* gene, so that in this case the protein was produced as a β -galactosidase fusion protein. The first 29 amino acid residues are shown to correspond to the β -galactosidase (see Figure 6.18). It was noticed that the subclone P3C1/*Cla* I in which the pBluescript II KS (-) vector carried a 3.2 kb DNA fragment had lost the *Cla* I cleavage site (ATCGAT) so that it was no longer cut with *Cla* I after re-ligation. This might be explained as a proofreading mechanism in the *E. coli* cell. In the case that one or some nucleotides at the *Cla* I site was/were missing or a structural change occurred during digestion process, this site might be removed by the action of DNA polymerase I if a mismatch was present. This may have allowed by chance the chitinase sequence to be put in the same open reading frame as the β -galactosidase gene.

Expression under its own promoter of undigested clone P3C1 was proved by a higher quantity of the 95 kDa protein detected on SDS-PAGE after induction with chitin in the growth medium but no significant difference in quantity of the protein detected with induction by IPTG. On the other hand, expression of subclone P3C1/*Cla*I under the control of *Plac* promoter was proved by a huge amount of the 95 kDa protein after induction with IPTG compared with no induction. In addition, even though the

cells were grown under induction of swollen chitin, only low expression was detected (see Figure 6.19)

6.7.4 Restriction Analysis of P1C1 and P3C1

Among approximately 2,100 transformants, eight clones were found to carry the chitinase gene and were isolated. All of the positive clones appeared to be identical based on *EcoR* I digestion patterns except P3C1. In addition, restriction-mapping analysis of clone P1C1 that represented all other clones and clone P3C1 were similar in their restriction patterns but different in the size of DNA insert. P1C1 carried a bigger DNA insert (5.5 kb) than that carried by P3C1 (4.0 kb) but both showed approximately 3.5 kb in the middle of their DNA insert which overlapped each other. In consideration of the restriction patterns, deletion analysis with three different enzymes (*Cla* I, *Sal* I and *EcoR* I) and expression of chitinase by Western blot analysis, it was concluded that the overlapping part of both clones contained the region encoding the same single chitinase gene. The localisation of the gene in the DNA insert of P3C1 was proposed (based on all the information given at this stage) to be between the *Cla* I site in DNA insert and 0.5 kb from the downstream end as highlighted in Figure 6.14A. However, additional information provided from the entire nucleotide sequence of the DNA insert of subclone P3C1/*Cla* I revealed that it lacked a few hundred nucleotides at the upstream part of the gene, including chitinase a control region and the sequence encoding some *N*-terminal amino acids. As a consequence, the entire gene is actually located a few hundred nucleotides upstream of the *Cla* I site.

6.7.5 Sequence Analysis of the Entire Chitinase Gene

The entire chitinase gene was constructed by joining the upstream portion that was missing in subclone P3C1/*Cla* I, but discovered in the original undigested clone P3C1, with the rest of the gene sequenced from subclone P3C1/*Cla* I. The candidate promoter was chosen based on highly conserved promoters discovered from more than 100 promoters from *E. coli* previously reported (McClure, 1985). The consensus

sequence at -10 region (Pribnow box) was found to be identical to that in the *E. coli lac* promoter. The distance between -35 and -10 (24 bp) was revealed to be longer than ordinarily suggested (17 ± 1 bp) (McClure, 1985). A Shine-Dalgarno sequence (AGGA) was found to be identical to that proposed for *Enterobacter agglomurans* (Chernin *et al.*, 1997). The signal sequence found here contained 21 amino acids, and the centre of the sequence comprised a highly hydrophobic stretch with alanine, valine, and leucine as major amino acids. All those were postulated previously to be the most common features for a typical signal sequence in both prokaryotic and eukaryotic genes (Stryer, 1995). The small, neutral amino acid residue alanine found on the amino-terminal side of the cleavage site was also found in other bacterial chitinases reported previously (Tsujibo *et al.*, 1993; Ueda *et al.*, 1994; Chernin *et al.*, 1997; and Tsujibo *et al.*, 1998) and suggested to be most common to be found in various genes. In addition, the presumption that the putative site of cleavage might be between alanine residues 21 and 22 is in good agreement with suggestions given by von Heijne (1983). The *N*-terminal sequence found from the DNA sequence coincided precisely with the sequence starting from alanine 21 and 22 (see Figure 6.20). It therefore corresponded with the amino acid sequence obtained from the protein sequencing. The codon TAA at nucleotides 2671-2673 was proposed to be a stop codon due to the presence of a self-complementary structure, inducing dissociation of the RNA-DNA hybrid, discovered 44 nucleotides downstream of the codon.

6.7.6 Localisation of the Recombinant Chitinase in *E. coli*

Western blot analysis indicated several different features between the recombinant chitinase expressed in *E. coli* XL1 Blue and the native chitinase from *V. carchariae*. It was found that the chitinase-positive clones produced a higher molecular mass (~95 kDa) compared to the wild type *V. carchariae* chitinase (63 kDa), indicating a precursor protein in *E. coli*. It was also found that *E. coli* synthesised and accumulated the 95 kDa chitinase inside the cells and formed inclusion bodies, while the *V. carchariae* chitinase was secreted into the growth medium. The location of the chitinase in inclusion bodies is different from previous studies on expression of

chitinases. In most cases, when genes encoding extracellular proteins are cloned in *E. coli*, the precursor is synthesised, processed, and exported across the inner membrane but not the outer membrane. For example, the clone carrying the chitinase gene from the marine bacterium *Alteromonas sp.* strain O-7 was expressed in *E. coli* JM109 by using pUC18. The cloned 85-kDa chitinase was not secreted into the growth medium but accumulated in the periplasmic space, while the native chitinase (ChiA) of *Alteromonas sp.* was recovered as a single 65 kDa polypeptide from the growth medium (Tsujibo *et al.*, 1992; Tsujibo *et al.*, 1993). However, the very compact and tightly folded structure of the protein in inclusion bodies was a possible explanation of why only very slight chitinase activity was detected in *E. coli*, even though a huge amount of the protein expressed was observed as shown in Western blot analysis.

There is no clear explanation how chitinases produced by marine bacteria are secreted outside the cells. However, extracellular secretion of several proteins produced by Gram-negative bacteria has been studied. It is believed that there are two main, highly conserved, pathways of secretions: (i) the signal peptide independent pathway, which is the one step mechanism of secretion of *E. coli* α -hemolysin (Holland *et al.*, 1990) and *Erwinia chrysanthemi* proteases A, B, and C (Ghigo and Wandersman, 1992), and proteins cross the cell envelope without a transient residence in the periplasm, (ii) the general secretion pathway (GSP), in which proteins such as *Klebsiella oxytoca* pullanase, cholera toxin (CT), and the structurally similar *E. coli* heat-labile enterotoxin (LT), cross the cytoplasmic and outer membranes in two distinct steps. In case of CT and LT, they consist of one A subunit and five identical B subunits. The A and B precursor polypeptides contain signal peptides that are cleaved off during export across the cytoplasmic membrane. Assembly of the subunits occurs in the periplasmic membrane. When assembled, both CT and LT are secreted from *V. cholerae*, but in *E. coli*, they remain in the periplasm (Hirst *et al.*, 1983; and Hofstra and Witholt, 1984 and 1985).

Mechanisms for extracellular transport of proteins have been identified in a diverse number of bacteria. It has been discovered that a cluster of genes that are homologous to the gene cluster of other secretory systems such as the *pul* cluster for

pullulanase (d'Enfert *et al.*, 1987) is involved in the general secretion pathway. For example, at least 12 genes are encoded by the gene (*eps*) cluster of *V. cholerae* (Overbye *et al.*, 1993; and Sandkvist *et al.*, 1997). It has been shown that the general secretion pathway is required for secretion of other proteins including chitinases through the outer membrane. Cloning and sequencing of a DNA fragment from *V. cholerae*, containing 12 open reading frames, *epsC*- to *-N* were reported. It was shown from their study that *eps* cluster encoded proteins essential for GSP function. Mutation in the *eps* genes resulted in aberrant outer membrane protein profiles, which indicated that the GSP, or at least some of its components, was required not only for secretion of soluble proteins but also for proper outer membrane assembly. In addition, immunoblotting experiments (Connell *et al.*, 1998) using M14, a *epsE* mutant of strain 569B, showed accumulation of ChiA in periplasmic fraction compared with the wild-type strain, which secreted the enzyme into the growth medium. Moreover, when M14 was complemented with pTDC*epsE*, a plasmid encoding a wild-type copy of the *epsE* gene of 569B, over 82% of total immunoreactive protein was located in the culture supernatant, indicating that ChiA is an extracellular *V. cholerae* protein which is secreted by the *eps* system.

6.7.7 Attempted processing study

Even though the 95 kDa protein was mostly found inside the cells, a very slight amount was also detected in the growth medium of *E. coli*. This occurrence was not as expected because a secreted enzyme should be processed to a smaller mature protein before secretion. Secretion of the recombinant chitinase was also confirmed by clear zones produced when the clones carrying chitinase gene were grown on chitin agar plate (see Figure 6.8). Transportation of precursor protein out of the cells prior to being processed is unlikely to occur.

It was demonstrated that there was no processing with the recombinant chitinase expressed in *E. coli* system. This may be that specific peptidases, which were lacking in this *E. coli* strain, were required for processing of a foreign protein produced in *E. coli*. The idea of specific enzymes was supported by a recent study (Gal *et al.*, 1998).

They found that when the purified 52 kDa chitinase from *Serratia marcescens* KCTC2172 and cloned in *E. coli* was incubated with the crude cellular extract of the *S. marcescens*, a 35 kDa chitinase was produced as a result of proteolytic activity from the crude enzymes of *S. marcescens*. They also found that the enzyme was stable against all other proteases (trypsin, chymotrypsin, and papain) from animal and plant sources. This indicates that the protease cleavage was bacterium-specific but it was not *S. marcescens*-specific since another study showed that *E. coli* transformants containing pCHI52 produced both 52 kDa and 35 kDa chitinase. Nevertheless, different results were found in this study. No change in protein patterns was observed either under denaturing or non-denaturing PAGE conditions when the cell extract of *E. coli* was incubated with the cell extract of *V. carchariae* even up to 21 h. Moreover, having detected a high molecular weight protein with exactly the same position as the 95 kDa recombinant protein of *E. coli*, in the cell extract of *V. carchariae* instead of 63 kDa processed chitinase clearly suggested no processing occurred inside the cells of *V. carchariae*.

Processing of a chitinolytic enzyme, chitobiase from *V. harveyi* was suggested to occur at the cell membrane as the enzyme was transported to the outer membrane of the *E. coli* cells (Jannatipour *et al.*, 1987). No clear evidence exists to indicate where the processing of the chitinase enzyme occurs in *E. coli* but it was proposed that processing occurs at the cell membrane simultaneously with secretion as mentioned previously (see section 6.7.6).

Chapter 7

General Conclusions and Discussion

7.1 Evaluation of Assay Methods for Chitinase Activity

Three different assay methods; radioactive assay using [^3H]chitin, viscometric assay using glycol-chitin, and colorimetric assay using chitin-azure, were evaluated for the determination of activity of a commercially available chitinase from *Serratia marcescens*. Even though the high cost of substrate preparation and more caution in handling with radioactive material during the assay were required, the radioactive assay appeared to be the most reliable and sensitive method compared with the others. The minimum requirement for the amount of the enzyme with a good accuracy was 1 mU. Not only a higher amount of the enzyme was required for the viscosity assay, it was also not considered to be very practical when a large number of protein samples needed to be determined. Colorimetric assay using chitin-azure was not able to be performed successfully in this study due to no specificity of the enzyme towards the substrate. In other words, no degradation of the chitin-azure substrate by action of the enzyme was observed at all, even though a high amount of the enzyme was used (up to 16 mU) with a long period of incubation time (8 h). Because of reasons given above, the radioactive assay was considered to be the most effective method so that it was therefore chosen for further investigation of the chitinase activity.

7.2 Expression of Chitinases in Marine Bacteria, *Vibrios*

Two strains of marine *Vibrio*, *Vibrio alginolyticus* strain ATCC17749 and *Vibrio harveyi* strain ATCC14126, were initially investigated and found to have some chitinase activity. They were used as the chitinase source for preliminary studies on the effect of incubation time and effect of chitin induction on the expression of the

chitinase. These conditions were optimised in order to obtain the highest expression of the enzyme for isolation and purification purposes. It was concluded from both studies that expression for both *Vibrio* strains increased remarkably with time. The highest chitinase activity was detected when the growth of the cells was reaching their maximum (at four days of incubation for *V. alginolyticus* and three days of incubation for *V. harveyi*). In addition, higher chitinase activity was observed when chitin was added to the growth medium, indicating that expression of the gene encoding chitinase was inducible. This result corresponded to previous studies (Monreal and Reese, 1968; Huang *et al.*, 1996; Woo *et al.*, 1996; and Svitil *et al.*, 1997). It was however found that different forms of chitin had different effects on chitinase production. Higher activity was detected when the cells were grown with swollen chitin compared with when the cells were grown with flake chitin. Presumably, the more open structure of swollen chitin (acid treated) promoted the accessibility of the enzyme to the substrate molecules. As a consequence, it allowed the enzyme to degrade the substrate and the small soluble molecules were taken up more efficiently than non-treated chitin flakes. However, the mechanism of chitin induction at the DNA level is still unknown.

It has been suggested previously that bacteria normally secrete more than one form of chitinase (Watanabe *et al.*, 1990; and Svityl *et al.*, 1997). The chitinase activity measured here reflected accumulated forms of all the enzymes secreted into the cell culture.

Subsequently, fourteen more species of *Vibrio* were obtained and investigated for chitinase activity. This revealed that different *Vibrio* species secreted strikingly different levels of chitinases. Moreover, different expression levels were detected for the same *Vibrio* species but different strain. For example, *V. alginolyticus* strain 283 expressed the highest chitinase activity (0.78 Umg^{-1}), while *V. alginolyticus* strain 284 expressed a relatively low amount of the enzyme (0.1 Umg^{-1}). High chitinase activity was also observed with *V. carchariae* (0.55 Umg^{-1}) after 1 day of incubation. Expression of chitinases was also investigated by observation of clear zones, which were produced as a result of enzyme action on a chitin substrate, around single

Vibrio colonies on chitin agar plates. In comparison, expression levels detected by both types of assay corresponded well for each species.

7.3 Purification and Characterisation of a Chitinase from *Vibrio carchariae*

Protein purification was found to be a rate-limiting step in this study. First of all, *V. alginolyticus* was thought to be the most suitable source for isolation and purification purposes at the initial stage. Development of a purification scheme for *V. alginolyticus* chitinase was therefore attempted with three different conventional steps (ammonium sulphate precipitation, anion-exchange chromatography on DEAE-Sephadex A-50, and chitin affinity chromatography). However, it was eventually realised that this *Vibrio* secreted more than one form of chitinase with differences in molecular size as proved on SDS-PAGE and Western blotting (Figure 4.10) in to the growth medium. Moreover, not only were chitinases produced, but the cells also secreted a large number of non-chitinase proteins as noticed from protein patterns of crude extracts analysed on SDS-PAGE (data not shown). As a result, the attempted purification for *V. alginolyticus* was found to be difficult to achieve.

After purification of *V. alginolyticus* was not performed successfully, another species, *V. carchariae*, was then considered and it was found to be a more suitable source for protein purification as only a single form of chitinase was produced by this bacterium. Moreover, the bacterium appeared to secrete fewer total proteins into the growth medium, which encouraged the possibility of obtaining the purified enzyme from this source. A purification scheme of a *V. carchariae* chitinase was developed successfully with three purification steps: chitin affinity chromatography, followed by gel filtration on Sephacryl-S200 HR, and Mono Q FPLC.

Chitin affinity chromatography proved to be a high performance step in terms of removal of a huge amount of undesired contaminating proteins in the starting culture. Nevertheless, oligosaccharide fragments occurred as a result of incomplete degradation of the chitin resin by the action of the enzyme. These were found to bind very strongly to the enzyme molecules and caused heterogeneity in charge and size

of the enzyme. As a consequence, it made further purification steps much more difficult. A lot of effort was put in to overcome the difficulties of oligosaccharide binding. Different chromatographic steps (mainly gel filtration on Sephacryl-S-200 HR and Superose®12 FPLC) were tested but they were never successful until anion exchange chromatography using Mono Q FPLC was replaced the Superose step.

The presence of acid hydrolysed monosaccharide (glucosamine) determined by quantitative analysis using the PAHBAH reaction and qualitative analysis on paper electrophoresis and paper chromatography confirmed contamination of carbohydrate in the enzyme preparation. It appeared that if the enzyme solution was left standing at room temperature after chitin affinity step, it helped the enzyme to cleave the chitin fragments that attached to the enzyme molecules completely.

Another problem that was encountered during protein the purification step using chitin affinity chromatography was to obtain specific elution conditions. It was discovered that *N*-acetylglucosamine (GlcNAc), which is supposed to be the final product of enzyme degradation, was not a good candidate for enzyme elution. The result also indicated that the enzyme appeared to bind so tightly to the chitin that no mild conditions tested could be used. Even though strong conditions: 5% (v/v) acetic acid, 15% (v/v) acetic acid or 6 M guanidine HCl were able to elute the enzyme, the results were disappointing as no enzyme activity was recovered after the elution. An only condition that proved to be acceptable in this study was to use 2 M guanidine HCl with 30% activity remaining after elution.

In summary, a complete purification of a chitinase from *V. carchariae* showed a single band of protein at 63 kDa on SDS-PAGE. According to the native molecular mass of the enzyme (66 kDa) determined by gel filtration on Sephacryl S200-HR, the enzyme contains a single subunit, and was thus similar to the chitinase purified from various other bacterial sources (Fukasawa *et al.*, 1992; Murao *et al.*, 1992; Tsujibo *et al.*, 1992; and Hiraga *et al.*, 1997). However, three major protein bands that possessed chitinase activity, were observed when the purified protein was analysed under non-denaturing conditions. This was explained as a result of deamidation

process or possible carbohydrate attachment, thus causing heterogeneity in the protein molecules. Over all, approximately 4.0 mg protein was purified from 670 mg total proteins in 1900-ml starting culture. After the final purification step (Mono Q fplc), the specific activity was 4.9 Umg⁻¹ with 7.8 fold purification. However, the low yield (4.5% recovery) remained because of the loss of enzyme activity that occurred when the protein solution was allowed to stand for a few days at room temperature to complete the action of the enzyme on the chitin molecules.

Once the purified *V. carchariae* chitinase was obtained, it was investigated for its *N*-terminal amino acid sequence. Sixteen amino acid residues (APTAPSIDMYGSNNLQ) were sequenced and appeared to show homology with the *N*-terminal amino acid sequence of ChiA from soil borne bacterium *Serratia marcescens* (Jones *et al.*, 1986) and from *Enterobacter agglomerans* (Chernin *et al.*, 1997), and ChiA from marine bacteria *Alteromonas sp* strain O-7 (Tsujibo *et al.*, 1992). The multiple alignment showed that the *Vibrio carchariae* sequence is about 50% identical to other chitinases (see section 7.7), but comparing just at the *N*-terminal region, it shows 25% identity with the residues that were completely conserved for all the sequences (see chapter 4, section 4.6.3). This suggested that the *V. carchariae* chitinase might be classified as belonging to the same chitinase group (Chi A). In addition, the compared sequences showed a complete conserved amino acid residues at three positions: alanine at the first position, proline at position two and five. In addition, a highly conserved amino acid isoleucine at position seven from the *N*-terminus. However, the *V. carchariae* *N*-terminal sequence showed no sequence similarity with the published sequence of *V. harveyi* chitinase (from SWISS-PROT, accession U81496; Svitil and Kirchman, 1996). This possibly implied that the chitinases from different groups were being studied. It is known that *V. harveyi* secretes more than ten chitinases. Some of which showed quite different properties (Svityl *et al.*, 1997).

Polyclonal antibodies raised against the purified *V. carchariae* chitinase were also prepared for gene isolation purposes. The anti-chitinase serum was produced with a very high titre and high specificity based on Western blot analysis. No cross-reaction

of the antiserum with other proteins in the crude solution of *V. carchariae* was observed, but it showed a very strong signal with a single 63 kDa chitinase band, even though the anti serum was diluted up to 20,000 times. This was also a clear indication that a single form of chitinase was secreted by *V. carchariae*. Moreover, the antiserum cross-reacted strongly with a broad range of protein bands (between 45-97 kDa) from crude enzymes of *V. alginolyticus*, indicating closely related of these proteins to the *V. carchariae* chitinase. It was believed that all these protein bands were multiple forms of chitinases secreted by *V. alginolyticus* with a high similarity to the *V. carchariae* chitinase. The anti-chitinase serum responded weakly with two protein bands from crude enzymes of *V. campbellii*, but no cross-reaction with crude enzymes from the other *Vibrio* species tested was observed.

7.4 Attempted Development of Nucleic Acid Probes

An attempt to develop nucleic acid probes for genomic library screening by the PCR technique was unsuccessful. The PCR reaction was performed using degenerate nucleotide primers designed based on the *N*-terminal amino acid sequence of the *V. carchariae* chitinase. Nucleotide sequence analysis of the PCR products proved that they were a consequence of non-specific annealing between the oligonucleotides and *EcoR* I digested genomic DNA fragments. The oligonucleotide primers used in this experiment were not considered to be optimal since they were designed based on amino acid residues that contained rather high codon degeneracy value. It was likely that these primers were competing with each other to bind to the DNA templates and because of variation in their sequences, it was possible for each sequence to anneal non-specifically to any DNA templates that showed some similarity to the *V. carchariae* chitinase sequence.

Using oligonucleotide probe for the genomic library screening was proved to be unsuccessful so that an immunological method was tried.

7.5 Isolation of Gene Encoding Chitinase

A genomic library was prepared by partially digested genomic DNA of *V. carchariae* with *Sau3A* I in order to isolate the gene encoding chitinase gene. Fragments from the *Sau3A* I partial digests (4-7 kb) were ligated to *BamH* I site arms of the pBluescript vector and transformed into *E. coli* XL1 Blue. Ligation efficiency obtained in this study was found to be 41%. The genomic library was screened by two different methods: immunological assay using anti-chitinase serum raised against the *V. carchariae* chitinase, and chitinase activity assay on chitin agar plates. With regard to immunological assay using the Western blot technique, twelve clones responded to the anti-chitinase serum among approximately 2,100 clones carrying DNA inserts obtained in the primary screening. Only eight clones remained active in the secondary screening. All these clones were tested for chitinase expression on a chitin agar plate. The chitin plate assay, by observation of clear zones produced around the colonies by action of the enzyme secreted to the agar medium, revealed that different anti-chitinase serum positive clones expressed different levels of chitinase. The clone designated "P3C1" showed the highest activity compared to the others.

All the chitinase positive clones, except P3C1, exhibited the same patterns of *EcoR* I digestion. Therefore, they were likely to be identical, and clone P1C1 was chosen for further investigation by restriction mapping. The most chitinase active clone P3C1 showed a slightly different *EcoR* I digestion pattern, so that it was presumed to be different. With regard to restriction mapping of clones P1C1 and P3C1 determined by ten restriction enzymes including *Kpn* I, *Sal* I, *Cla* I, *Pst* I, *Spe* I, *Xba* I, *EcoR* I, *Hind* III, *Sau3A* I, and *Xho* I, the clone P1C1 appeared to contain a bigger DNA fragment (5.5 kb) compared with the clone P3C1 (4.0kb). Both showed similarities in their restriction patterns. Since it carried a smaller DNA fragment with the highest chitinase activity detected, the clone P3C1 was therefore considered to be a suitable clone for further studies.

Deletion analysis of the clone P3C1 with three different enzymes (*Cla* I, *Sal* I, and *EcoR* I) was performed prior to beginning the sequencing experiment in order to localise the chitinase gene within the DNA insert. By consideration of the full length of the protein (95 kDa) that responded to the anti-chitinase serum and the intensity of signals detected from the colony by Western blot technique, the *Cla* I digested clone (designated P3C1/*Cla* I), in which some upstream part of DNA fragment (approximately 800 bp) was removed, still appeared to fully express recombinant chitinase. It was therefore assumed at that stage that subclone P3C1/*Cla* I contained a 3.2 kb DNA insert with a full length of a chitinase gene and was suitable for determination of the nucleotide sequence of the whole DNA insert.

The double-stranded DNA fragment (4,381 bp) was automatically sequenced by Oswel. Computer analysis of the obtained sequence showed that the *Cla* I digested clone contained a DNA insert with a single reading frame encoding 820 amino acids corresponding to chitinase. The sequence however lacked the upstream part of the gene including the control expression regions i.e. promoter, transcriptional starting site, and ribosome binding site, and the sequence responsible for encoding a signal sequence and some amino acid residues at the *N*-terminus.

It was also discovered that chitinase expression of the subclone P3C1/*Cla* I was induced by IPTG, indicating that the gene was expressed under the control of the *Plac* promoter as a fusion protein. However, the important upstream part of the gene that was lacking in the *Cla* I digested clone was discovered from the undigested clone (P3C1), and a complete sequence of the chitinase gene was thereby obtained.

The sequence contained a single reading frame (2,550 bp) encoding 850 amino acid with a calculated molecular weight of 90,249. The promoter regions at -35 and -10 were 24 bp far apart and proposed to be TTACAC and TATGTT, respectively. The sequence at -10 region (Pribnow box) was discovered to be identical to that in the *E. coli lac* promoter (*Plac*). The Shine-Dalgarno sequence (AGGA) was investigated and found to be identical to that proposed for *Enterobacter agglomurans* (Chernin *et al.*, 1997). The secretion signal sequence found here contained 21 amino acid

residues with a highly hydrophobic stretch with alanine, valine, and leucine as major amino acids. The putative cleavage site was between alanine residues 21 and 22. The stop codon TAA was found at the downstream end of the gene (at nucleotides 2,671-2,673) and the inverted repeat sequence (AAAAGGACAGC....GCTGTCCTTTT) was found 44 nucleotides beyond the stop codon. The sequence indicates where transcribed mRNA would form a self-complementary structure, and dissociation of the mRNA from DNA template would subsequently occur.

7.6 Expression of the Recombinant Chitinase in *E. coli*

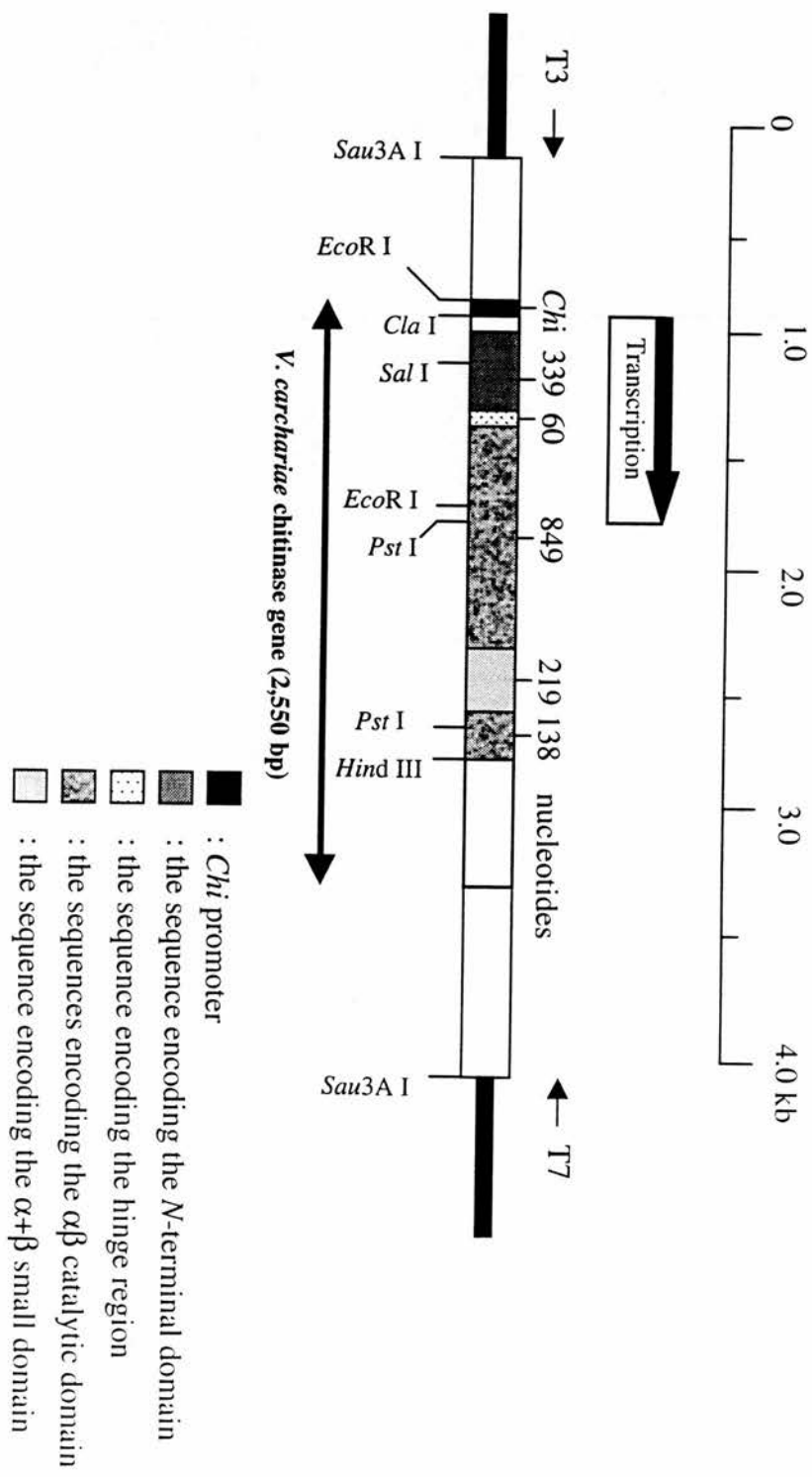
Even though the positive colonies showed strong signals of anti-chitinase serum-chitinase complexes, expression levels of chitinase detected by chitin plate assay were much less than the native chitinase secreted by *V. carchariae*. It was proved by Western blot analysis that all the clones expressed the precursor protein with higher molecular mass (95 kDa) compared to the native protein (63 kDa). Moreover, very little amount of the protein was detected in either cell culture or cell extract but was found in inclusion bodies. This was different from a previous study on *Alteromonas* sp. strain O-7, that the cloned chitinases were detected in the periplasmic space of the cells.

With regard to the clone P3C1 that was chosen for determination of the nucleotide sequence of the chitinase gene, it was found that levels of chitinase expression was dramatically promoted by swollen chitin added to the growth medium. This suggested that the chitinase gene was expressed under the *Vibrio* chitinase promoter. This also implied that the *Vibrio* promoter functioned well in an *E. coli* system.

Based on the entire nucleotide sequence information and deletion analysis, the chitinase gene in the 4.0 kb DNA insert was found to be located approximately 700 bp from the upstream end and 800 bp from the downstream end of the DNA insert (Figure 7.1). The calculated molecular mass (90 kDa) of the precursor chitinase was slightly smaller than that estimated on SDS-PAGE (~95 kDa). The high molecular weight precursor protein was similar to that reported for *V. parahaemolyticus*, in

Figure 7.1 Localisation of the chitinase gene in a 4.0 kb DNA insert of the clone P3C1

The chitinase gene in the DNA insert is indicated. The regions corresponding to the chitinase (*Chi*) promoter, *N*-terminal domain, hinge region, α/β catalytic domain, and $\alpha+\beta$ insertion domain (as discussed later in section 7.7) are highlighted.



which case the chitinase gene cloned into pBR322 and transformed to *E. coli* DH1 produced a 95 kDa chitinase (Laine *et al.*, 1988). However, they suggested that the 95 kDa cloned chitinase corresponded well with the size of the chitinase produced by the native *Vibrio* strain, indicating that the protein is not processed.

Apart from the case for *V. parahaemolyticus*, the cloned chitinase expressed in this study appears to be the biggest precursor protein ever reported. For example, two cloned chitinases from *Alteromonas sp* strain O-7 were expressed with differences in their molecular sizes (85 kDa and 78 kDa). They suggested that the 78 kDa chitinase was derived from the 85 kDa chitinase as a result of proteolytic cleavage (Tsuijibo *et al.*, 1993). A pCHI52 clone carrying a chitinase gene isolated from *Serratia marcescens* strain KCTC2172 secreted a 52 kDa precursor chitinase and a 35 kDa chitinase. It was also suggested from the amino acid sequence identity that the 35 kDa protein was a result of a post-translational proteolytic modification (Gal *et al.*, 1998).

The protein processing that occurred in *E. coli* reported from the two studies mentioned above was different from that found in this study. Moreover, it was confirmed here that *E. coli* XL1 Blue does not have the proteolytic enzyme involved in chitinase processing. It also proved that the protein processing does not occur inside the cells. Indication of non-processing protein produced and accumulation of the protein in inclusion bodies would possibly be a clear explanation of small chitinase activity of the enzyme detected from clones carrying chitinase gene.

7.7 Prediction of the Three Dimensional Structure of *V. carchariae* Chitinase

Once the entire sequence of the chitinase gene was determined and analysed, its amino acid sequence was translated and compared the amino acid sequences in databases. Searching results revealed that the *V. carchariae* chitinase showed high similarity with all ChiA enzymes from databases with the highest similarity with a ChiA from *Alteromonas sp.* strain O-7 (54% identity), followed by ChiA from *Enteromonas agglomerans* (53% identity), from *Serratia marcescens* (52% identity),

and from *Aeromonas caviae* (49% identity). The protein showed moderate similarity with ChiA from *Bacillus circulans* (31% identity). It was also showed that the amino acids sequence This suggests that the chitinase from *V. carchariae* belongs to the same group of enzyme. However, relatively low similarity with ChiA from *Vibrio harveyi* (only 19% identity) was found. Presumably these two enzymes were derived from different ancestral genes. The fact that the name of ChiA does not belong to the same subgroup was discussed in chapter 1, section 1.2.

The multiple alignment of five closely related chitinases from bacterial sources is shown in Figure 7.2. The multiple alignment shows that these amino acid residues near the *N*-terminus of the *V. carchariae* chitinase are different from all other sequences. Highly conserved regions are found between amino acid residues 257-281, amino acid residues 289-320, amino acid residues 352-367, amino acid residues 283-395, and amino acid residues 448-465. These regions are shown to be important in the enzyme function because they are at the active site.

A model of the *V. carchariae* chitinase three dimensional structure was built based on the X-ray structure of ChiA from *S. marcescens* (Perrakis *et al.*, 1994). The structured-based alignment (Figure 7.3) was made using "CLUSTALW" algorithm in the GCG package (Thompson *et al.*, 1994), and the model was built up using the program called "O" (Jones *et al.*, 1991). In principle, the amino acid replacement was used to mutate the structure of *S. marcescens* ChiA for *V. carchariae* ChiA. If the new side chain was similar or smaller in size, it was followed as closely as possible the conformation of the original side chain. If the new side chain was larger than the original side chain, it was put in a rotamer library (Jones *et al.*, 1991), and the rotamer conformation that showed most favourable for all other structures, and did not clash with the rest of the protein was used.

After all the 'the easy' residues were built in, we expected to see some differences between the modelled structure and the crystal structure at some interesting places i.e. active site. However, it became apparent that the structures would be very similar, and that all the important features were conserved. Therefore, it did not

Figure 7.2 Multiple alignment of deduced amino acids sequences of some bacterial chitinases

The amino acid sequences of chitinases from *V. carchariae* (chiA_Vcar, deduced from translation of the DNA sequence obtained in this study), *Alteromonas sp.* strain O-7 (chiA_Altso, ChiA_Altso from the Swiss-Prot database), *Serratia marcescens* (chiA_Smar, ChiA_Serma from the Swiss-Prot database), *Enteromonas agglomerans* (chiA_Eagg, OWL:EAU59304 from the Swiss-Prot database), *Aeromonas caviae* (chiA_Acav, OWL:ACU09139 from the Swiss-Prot database), and *Bacillus circulans* (chiA_Bcir, Chi1_Bacci from the Swiss-Prot database) are aligned using "CLUSTALW" algorithm of the GCG package. The amino acids that are identical are marked with asterisks. Amino acids that are similar are shown with one or two dots.

chiA_Vcar	MIRFNLCAAGVALALSGAANAAPTAPSIDMYGSNNLQFSKIELAMETTSQGYNDMVKYHEL	60
chiA_Altso	-----	
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	-----	
chiA_Bcir	-----	
chiA_Vcar	AKIKVKFNQWSGTSBGDTYNVYFDGVKVATGAITG--SQTTASFEYQGQGLYQMEIEACDA	118
chiA_Altso	---SIKWNASGSGGDNYKVYFDDLLVNQGSPLAGTKSGVVQFPYTKSGRHLQYLELCEG	57
chiA_Smar	-DVSVSWNLWNGDAGTTAKILLNGKEAWSGPSTG--SSGTANFKVNKGGRYQMQUALCNA	57
chiA_Eagg	-DVSISWDLWSGDAGTTAKVLLDGKEVWSGASTG--TSGTANFKVNKGGRYQMQUALCNA	57
chiA_Acav	APVSVTWNLWSGDVGQTAKVLLDVKEVWSGPAS---AAGTANFKVTKGGRYQMQUALCNA	57
chiA_Bcir	-----	
chiA_Vcar	TGCSKSAPVEITIAADTDGSHLKPLTMNVDPNNKSYNTDPSIVMGTYFVEWGIYGRDYTVD	178
chiA_Altso	TVCARSAGKEIVIAADTDGAHLAPLPMNVDPNNRNNGTIPGRVTGAYFVEWGIYGRNYDVT	117
chiA_Smar	DGCTASDATEIVVADTDGSHLAPLKEPLLEKNKPYKQNSGKVVGSYFVEWGVYGRNFTVD	117
chiA_Eagg	DGCTASDVTQIVVADTDGSHLAPLKEPLLENNKPYKQDSGKVVGSYFVEWGVYGRNFTVD	117
chiA_Acav	DGCTLSDDKELMVADTDGSHLAPLNAPLKNNKPYANKSGKVVGAYYVEWGVYGRKFTVD	117
chiA_Bcir	-----NVA	3
	*	
chiA_Vcar	NMPVDNLTHILYGFIPICGP---NESVKS VGG-N--SFNALQTACRGVNDYEVVIHDPWA	232
chiA_Altso	KIPAHNLSHILYGFIPICGP---NESLKSIEIGN--SWRALQTACADSQDYEVVIHDPWA	172
chiA_Smar	KIPAQNLTHLLYGFIPICGGNGINDSLKEIEG---SFQALQRSCQGREDFKVS IHDPFA	173
chiA_Eagg	KIPAQNLTHLLYGFIPVCGGDGINDSLKGVEG---SFQALQRSCQGREDFKVS IHDPFA	173
chiA_Acav	KIPAQNLTHILYGFIPICGGNGINDSLKEISG---SFEALQRSCAGREDFKVS IHDPWA	173
chiA_Bcir	DIDPTKVTHINYAFADICWNGIHGNPDPSGPNPVTWTCQNEKSQTINVPNGTIVLGDPWI	63
	.. : : : * . * : * . . : . : . : : * :	
chiA_Vcar	AYQKSFPQAGHEYSTPIKGNYAMLMAKQRPDLKIIPSIGGWTLSDPFYDFVD-KKNRD	291
chiA_Altso	AVQKSM-PGVDAKD-PIRGVYSQLMALKQRYPDCLKILPSVGGWTLSDPFHGFTN-KANRD	229
chiA_Smar	ALQKAQ-KGVTAWDDPYKGNFGQLMAKQAHDPDLKILPSIGGWTLSDPFFFMGD-KVKRD	231
chiA_Eagg	AVQKGQ-KGVTAWDDPYKGNFGQLMAKQARPDLKILPSIGGWTLSDPFFFMGD-KVKRD	231
chiA_Acav	AIQMSQ-GNLSAWDEPYKGNFGNLMAKQAHDPDLKILPSVGGWTLSDPFYFLGD-KTKRD	231
chiA_Bcir	DTGKTF--AGDTWDQPIAGNINQLNKLKQTNPNLKTIIISVGGWTWSNRFSVAATAATRE	121
	. * * * * * : * : * * * * : * . . * :	
chiA_Vcar	TFVASVKKFLKTKWKFYDGVDDIDWEFPGGGGAAADKGDPVNDGPAYIALMREL RVMLDELE	351
chiA_Altso	TFVASVKQFLKTKWKFYDGVDDIDWEFPGGDGPNDLGDPINDGPAYVALMQELRAMLDELE	289
chiA_Smar	RFVGSVKEFLQTWKKFFDGVDDIDWEFPGGKGANPNLGSQP-DGETYVLLMKELRAMLDQLS	290
chiA_Eagg	RFVGSVKEFLQTWKKFFDGVDDIDWEFPGGGANPKLGNAQ-DGATYVQLMKDLRAMLDQLS	290
chiA_Acav	TFVASVKEFLQTWKKFFDGVDDIDWEFPGGQGANPSLGGPN-DGATYVVLMLKELRAMLDELE	290
chiA_Bcir	VFANSAVDFLRKYN-FDGVLDLWEYPVSGGLDGNNSKRPE-DKQNYTLLLSKIREKLDAAG	179
	* . * . * : : : : * : * : * : * . * . * * * : : * *	
chiA_Vcar	AETGRTYELTS AIGVG YDKIEDVDYADAVQYMDYIFAMTYDFYGGWN-NVPGHQ TALYCG	410
chiA_Altso	AETGRQYELTS AIGAGYDKIEDVDYQAAQYMDYIFAMTYDFYGAWN-NETGHQTGIYCG	348
chiA_Smar	AETGRKYELTS AISAGKD KIDKVAYNVAQNSMDHIFLMSYDFYGPFDLKNLGHQTALNAP	350
chiA_Eagg	AETGRKYELTS AISAGKD KIDKV DYN TAQNSMDHIFLMSYDFYGAFDLKNLGHQTALKAR	350
chiA_Acav	AETGRQYELTS AISAGGD KIAKV DYQAAQYMDHIFLMSYDFSGAFDLTNLAHQTNLFGS	350
chiA_Bcir	AVDGKKYLLTIASGASATYAANTELAKIAAIVDWINIMTYDFNGAWQ-KISAHNAPLN YD	238
	* * : * * * * . . : * * * : * * * : : . * : :	
chiA_Vcar	SFMRPGQCDGGGV DENGEPYKGPAYTADNGIQLLLAQGV PANKLV LGTAMYGRGWEGVTP	470
chiA_Altso	SHLSTDECNGTGVDDNGVPRKGPAYTGDHAIQLLLQQGVQPSKLVGMVAMYGRGWEGVLD	408
chiA_Smar	A-----WKPD TAYTTVNGVNALLAQGVKPGKVVG TAMYGRGWTGVNG	393
chiA_Eagg	P-----GNRHGLYHGER-RQCVAGQGVKPGKIVVGA AKYGRGWTGVSG	392
chiA_Acav	S-----WDPATKYTTDKGVKALLGQGVTPGKIVVGA AMYGRGWTGVKN	393
chiA_Bcir	PAASAAG-----VPDANTFNVAAGAQGHLDAGVPAAKLV LGVPFYGRGWDGCAQ	287
	. : : * * . * : * . * * * *	
chiA_Vcar	DTLTDPNDPMTGTATGKLKGSTAQGVWEDGVIDYKGIKSFMLGANNTGINGFEYGYDAQA	530
chiA_Altso	ANAAIPGNPMTAPGNGLPTGSTSEGVWEPGIMDYKAI AANAVGQGGSGVNGYEVGYDEQA	468
chiA_Smar	YQN---NIPFTGTATGPVKG-----TWKNGIVDYRQIAGQFMS-----GEWQYTYDATA	439

chiA_Eagg	YQN---NNPFTGTATGPVKG-----TWENGIVDYRQIANEFIS-----DEWQYSYDATA	438
chiA_Acav	YQA---GNPFTGTATGPVSG-----TWENGVDYRDIVNNRMG-----AGWEQGYDESA	439
chiA_Bcir	AGN---GQYQTCTGGSSVG-----TWEAGSFDYDLEANYINK-----NGYTRYWNDTA	333
	* * .*: * .*: : : : : *	
chiA_Vcar	EAPWVWNRSTGELITFDDHRSVLAKGNYAKSLGLAGLFSWEIDAD-NGDILNAMHEGMAG	589
chiA_Altso	QAAYVWNRNSGKLITYDSPRSVIAKGQYANTHQLAGLFGWEIDAD-NGDILNAMYDGL--	525
chiA_Smar	EAPYVFKPSTGDLITFDDARSVQAKGKYVLDKQLGGLFSWEIDAD-NGDILNSMNASLGN	498
chiA_Eagg	EAPYVFKPSTGDLITFDDPRSVQAKGKYVLDKQLGGLFSWEIDAD-NGDILNNMNTSLGN	497
chiA_Acav	EAPYVFKASSGDLITFDNDRSVKTKGQYVLANQLGGLFAWEIDAD-NGDILNAMHEGLGH	498
chiA_Bcir	KVPYLYNASNKRIFISYDDAESVGYKTAYIKSKGLGGAMFWELSGDRNKTQLQNKLKADLPT	393
	:.:.:.: * . :*: * .** * * * .*: :*:..* * : * : .:	
chiA_Vcar	GVVTPPNR--KPTAAAGADQAVTGPASVVLDSNSTSDGTIASYAWEQVSGTAVVLGSA	647
chiA_Altso	TAGEIPNR--APTIGVSGPINVTSGQVVNVDAQAS-DLDNDPLTYSWVAAPGLALSANN-	581
chiA_Smar	SAGVQPRE-----	506
chiA_Eagg	SAGAQPRE-----	505
chiA_Acav	GEGLTPANKPPVANAGSDLSATGPAEVTLNLSASHDPENGALTYSWKQVSGPQASLLDV	558
chiA_Bcir	GGTVPPVDTTAPSVPGNARSTGVTANSVTLAWNASTDNVGVGTG-YNVYNGANLATSVTG-	451
	*	
chiA_Vcar	NTATASFDAVEVAEEQLTFKLTVTDNEGATASDLVVVTVKPAVIDPPNTAPVAQVSAP	707
chiA_Altso	-TAAVAVTAPSPAQQTSYDLTVTVNDGALSTTKTIVVVVN-----PEGANAAPVVPVSD	635
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	TQAKARVVLDAVSSDINLVFELTVTDDQGLSAKDQVVVTNK---APQPNLPPVVSVPAS	614
chiA_Bcir	-----TTATISGLTAGTSYFTTIKAKDAAGNLSAASNAVTVSTTAQPGGDTQAPTAPTNL	507
chiA_Vcar	ATASAGDVVIDASASSDADNDTLTFDWTLPQGLN-ATVNGAKVTFTAEEYPQDTSLSFT	766
chiA_Altso	ISVNEGASATVNVSA-TDPEGAALSYSWSPAELSVANGSSAT--ITAANVTADTTVPVT	692
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	ATVEAGKQVSIKATA-SDPNGDALSYQWTVPAGLSATGLDSATLVVTGSNVTSDTVYDLT	673
chiA_Bcir	STAQTTSSITLSWTASTDNVGVGTGYDVYNGTALATTVTGTTAT---IS-GLAADTSYTFT	563
chiA_Vcar	VSVSDGQAVSTASATVVVAKHSTG-----GGTCTNAWDATAVYTGGDQVITYAGKTWE	818
chiA_Altso	VTVSDGVNAVDTTFNVTIK-----DGAEYPTWDRSTVYVGGDRVIHNSNVFE	739
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	LVVTDGALDATAVTRLIVKPASTGGGCEASDPDAANHPAWSAGTVYNTNDKVSHKQLVWQ	733
chiA_Bcir	VKAKDAAGNVSAASNAVSVKTAET-----TNPGVSAWQVNTAYTAGQLVITYNGKTYK	616
chiA_Vcar	AKWWDsrvKIL--ASLANGVFGKKLVHLLVTKHC-----	850
chiA_Altso	AKWWTQGEPEG-TADVWKAVTNPRE-----	763
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	AKYWTQGNESRTADQWKLVSVQVLGWDAGVVYNGGDTVSHNGRKWKAQYWTGDEPGKA	793
chiA_Bcir	CLQPHTSLAGWEPSNPALWQLQPRE-----	642
chiA_Vcar	-----	
chiA_Altso	-----	
chiA_Smar	-----	
chiA_Eagg	-----	
chiA_Acav	AVWVDQGAASCNP	808
chiA_Bcir	-----	

Figure 7.3 A comparison of amino acid sequence of chitinase between *S. marcescens* chitinase and *V. carchariae*

This structure based-alignment was made by the program "CLUSTALW" (Thompson *et al.*, 1994). Amino acids that are identical are highlighted. Amino acids that are implicated as playing important roles are shown by asterisks.

Vcarchariae
Smarcescens

10 20 30
M I R E N L C A A G V A L A L S G A A N A A P T A P S T D M Y G S N N L Q F S
M R K E N K P L L A I L L I G S T L C S A A Q A A A P G K E T A W A G N T K F A

Vcarchariae
Smarcescens

40 50 60 70 80
K I L A M E T S G L N D M V K Y H E L A K I K V K F N Q S G T S G D T Y N V Y
I V L V D Q A A A A Y A N N L V K V K N A A D V S V S W N L N N G D A G T A K I L

Vcarchariae
Smarcescens

90 100 110 120
F D V K V A T A I T G S Q T A A S E Y G Q C G L V O M E I E A D A T G C S K
L N K E A W S P S A G S G A A N K V N K C G R V O M Q V A L N A D G C T A

Vcarchariae
Smarcescens

130 140 150 160
S A P V T I A D T D G S H L K E T M N V D P N V K S Y N T D P S I V M G T Y F
D A T L V V A D T D G S H L A E K E P L L E K K P K Q N S G K V V G S Y F

Vcarchariae
Smarcescens

170 180 190 200
D E A C I G C D Y V D N M V D V T H I L A G C S E S T C G P N E V K S
M R C V N F L K I A Q V T H I L A G C S E S T C G P N G I N D L K E

Vcarchariae
Smarcescens

210 220 230 240
V G N S E N A P O T A R C V N D Y E V V T H D P W A A Y O S F P O A G H E Y
I E S E Q A O R S Q R E F F K V S T H D P F A L O A K V T A W

Vcarchariae
Smarcescens

250 260 270 280
S T I K G N Y A M E A A K O R N E D L K I I S S G C W T L S D P F Y D F V D K
D D Y C N F G Q A A H E D L K I L S S G C W T L S D P F F F M G D K

Vcarchariae
Smarcescens

290 300 310 320
K N R I T P V A S Y K K E L K T W K F Y D G V D I D W E P P C G G A A A D K G D P
V K E R V G S Y K E E Q T W K F F D G V D I D W E P P C G K A N P N L G S F

Vcarchariae
Smarcescens

330 340 350 360 370
V N D C P A I A L M R S O U R V M L D E E E A R T G H T E E P S A T G V C Y D K T
Q E T V L K A A Q S A E N G K K A S A K D K T

Vcarchariae
Smarcescens

380 390 400
E D D A D V Q Y Y E A L T S D E A G W N N V P S E O T A Y C G S F
D K A N V Q N S H L L S S P F D L K N L S E O T A

Vcarchariae
Smarcescens

410 420 430
M R G Q C D G G G V D E N G E Y K G P A D A I Q L E A G G P A N L
N A G A W K D T T V V N A T D G G V K P G V

Vcarchariae
Smarcescens

440 450 460 470
L E I T V G I G E V T P D T L T D P D M C G A T K L G S T A Q G V
V E I G C A T N G Y Q N I F E G P V P V G

Vcarchariae
Smarcescens

480 490 500
E D V I D K G T K S F M L A N N T G I N G F E V G V D Q E A B W V W N R
K N I V R Q A Q F M S E W Q T Y D A T A A D Y F K P

Vcarchariae
Smarcescens

510 520 530 540
S L E D H A S L A K N A K S L G A A P S E P A D N G D A T
L D A Q A K V L D K Q G A P S E P A D N G D A T

Vcarchariae
Smarcescens

550 560
A H E G M A G G V V T P P N R K P T A A A G A D Q A V T G P A S V V L D G S N S
S N A S L G N S A G V Q

seem appropriate to embark on refining the model by building in the relatively small number of residues that required more extensive modelling.

From the modelled structure obtained (Figure 7.4), most of amino acid residues that are different from *Serratia* chitinase (shown in magenta) are on the outer surface of the whole molecule of the enzyme while the amino acid residues that are identical (shown in yellow) are buried inside. A space-filling model (Figure 7.5) shows a big groove that represents the active site of the enzyme with two amino acid residues Glu315 and Asp391 (shown in cyan). Glu315 is completely conserved in the family 18 chitinases and Asp391 is conserved in nine out of ten bacterial chitinases of family 18, and these residues play a very important role in enzyme catalysis.

The structure model of *V. carchariae* chitinase revealed high structural similarities to the *Serratia* chitinase as mentioned previously. The over all structure comprised three major domains: the amino-terminal chitin binding domain (residues 24-137), which consists only of β -strands, connects through a hinge region (residues 138-158) to the main α/β -barrel domain (residues 159-442 and 517-563). The third domain, which has an $\alpha+\beta$ fold, is formed by an insertion in the barrel motif (residues 443-516) to form the small domain (see ribbon diagram in Figure 7.6).

The amino-terminal domain was suggested to have some similarity to FnIII type domains that were located in some cellulases (Bèguin and Aubert, 1994). Even though little is known about how the *N*-terminal domain plays an important role in chitinase, it is believed that the domain is involved in interacting with the filamentous chitin substrate as discussed in section 1.7. It is noticed that carbohydrate-degrading enzymes such as cellulases, xylanases, and chitinases appear to share a common structural feature, which is reflected in the amino acid sequences of these enzymes. As reviewed by Gilkes *et al.* (1991a,b), analysis and comparison of the sequences of endo- β -1,4-glucanases CenA and Cex from *Cellulomonas fimi* have revealed conserved stretches that occur in discrete domains connected by linkers and allow the domains to function independently. The CBDs of Cex and CenA of *C. fimi* are 108 and 111 amino acids long, respectively, and their sequences

are more than 50% identical. Very similar sequences are found in other cellulases and xylanases (Gilkes *et al.*, 1991b). Functions of the carbohydrate-binding domain have been studied extensively in cellulases (β -1,4-glucanases). It has shown that some of cellulases comprise discrete catalytic domains and cellulose-binding domains (CBDs) which retain their function when separated by proteolysis. In both fungal and bacterial extracellular cellulases, the CBDs are believed to play a role in cellulose hydrolysis because catalytic activities are altered when they are deleted by proteolysis or genetic manipulation. Coutinho *et al.* (1993) showed that removal of CBD_{cenA} from endo- β -1,4-glucanase A (CenA), or its substitution by CBD_{cenC}, affected the catalytic activity of the enzyme on the various celluloses tested. However, it is suggested that although the CBDs are not involved directly in catalytic activity, they do modulate the specific activities of the enzyme on soluble and insoluble cellulosic substrates (reviewed by Gilkes *et al.*, 1991).

In bacteria, striking features of the CBDs sequences are; (i) low contents of charged amino acids; (ii) high contents of hydroxyamino acids; and (iii) conserved tryptophan, asparagine, and glycine residues. Furthermore, there are two cysteine residues in identical positions close to the *N*- and *C*-termini in all but one of the sequences and hydrophobic residues at 10 conserved sites in all of the sequences (Gilkes *et al.*, 1991b). Tryptophan residues are conserved in other types of polypeptides that interact with polysaccharides. One example can be found in the discrete starch-binding domains of microbial enzymes that degrade starch (Svensson *et al.*, 1989).

However, proteolytic removal of the *N*-terminal half of the CBD of CenA of *C. fimi*, which leaves only a single cysteine in the CBD, did not prevent the truncated enzyme from binding to cellulose, suggesting that a disulphide bridge between the two cysteines of the CBD is not essential for binding (Gilkes *et al.*, 1989). Moreover, it has been shown that CBDs, in which *N*- or *C*-terminal attached to catalytic domains, appear not to be determinants of specificities. An interesting example was provided by the endoglucanases from *Cellulomonas fimi* and *Microbispora bispora*, which

Figure 7.4 The wire model of chitinase from *V. carchariae*

The model was built up by the program called "O" (Jones *et al.*, 1991). Amino acids that are different from *S. marcescens* are presented in Magenta. Amino acids that are identical to *S. marcescens* are presented in yellow. Glu315 and Asp391 are shown in cyan. Nitrogen atom is presented in blue and oxygen atom is presented in red.

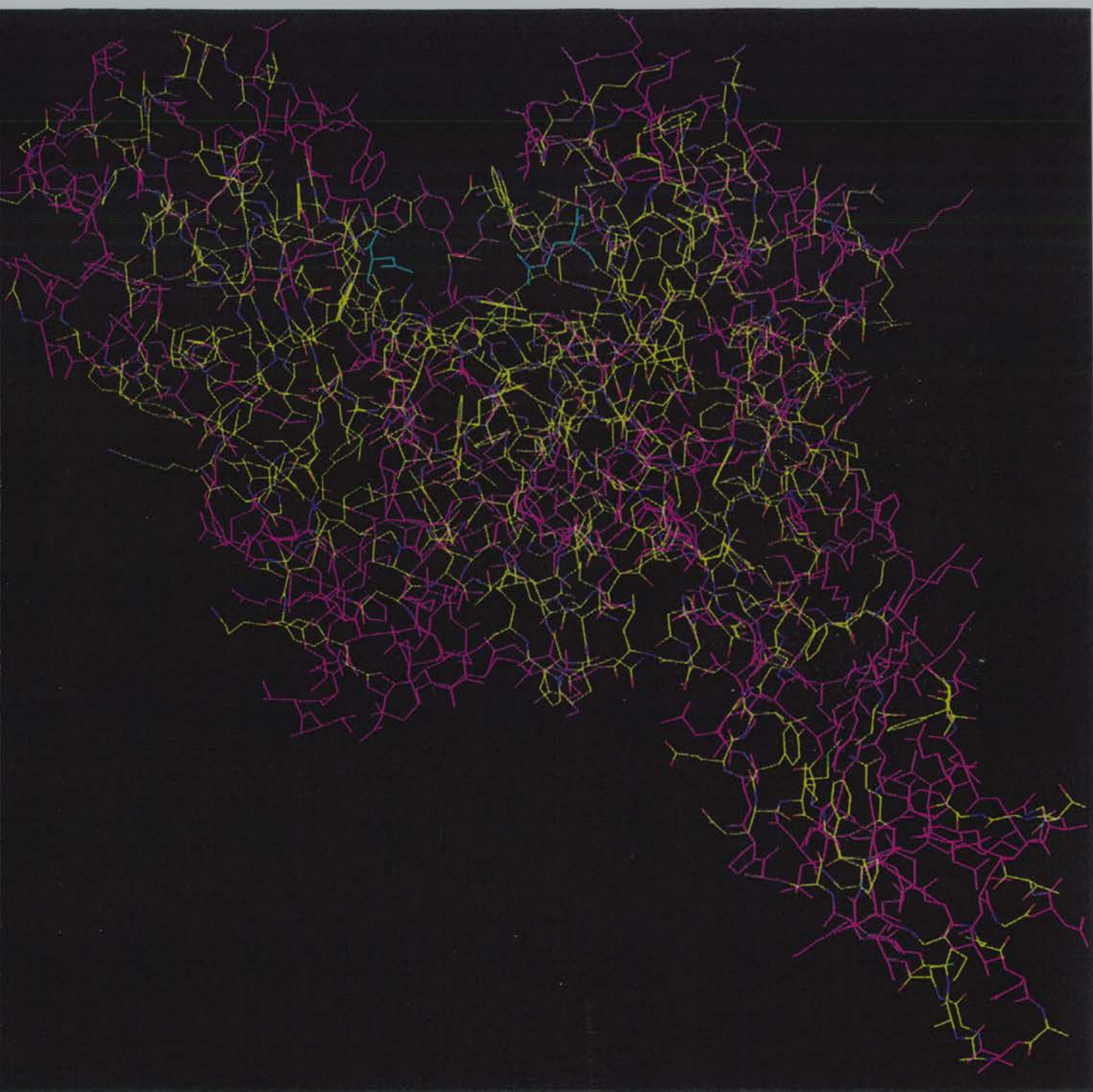


Figure 7.5 The space-filling model of chitinase from *V. carchariae*

The model was built up by the program called "O" (Jones *et al.*, 1991). Amino acids that are different from *S. marcescens* are presented in Magenta. Amino acids that are identical to *S. marcescens* are presented in yellow. The enzyme catalytic site is seen as a big cleft. Glu315 and Asp391 located in the cleft are shown in cyan. Nitrogen atom is presented in blue and oxygen atom is presented in red.

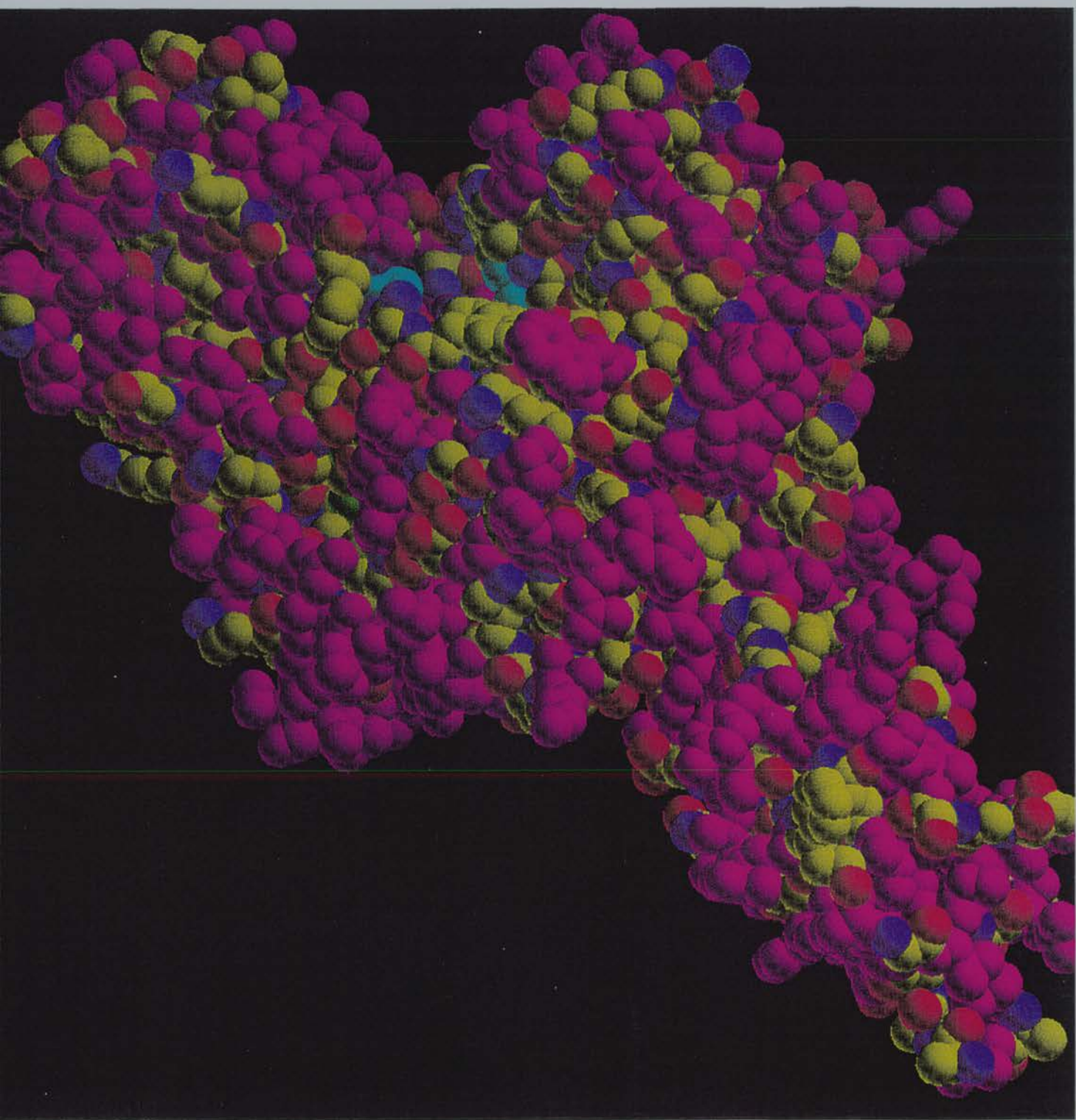
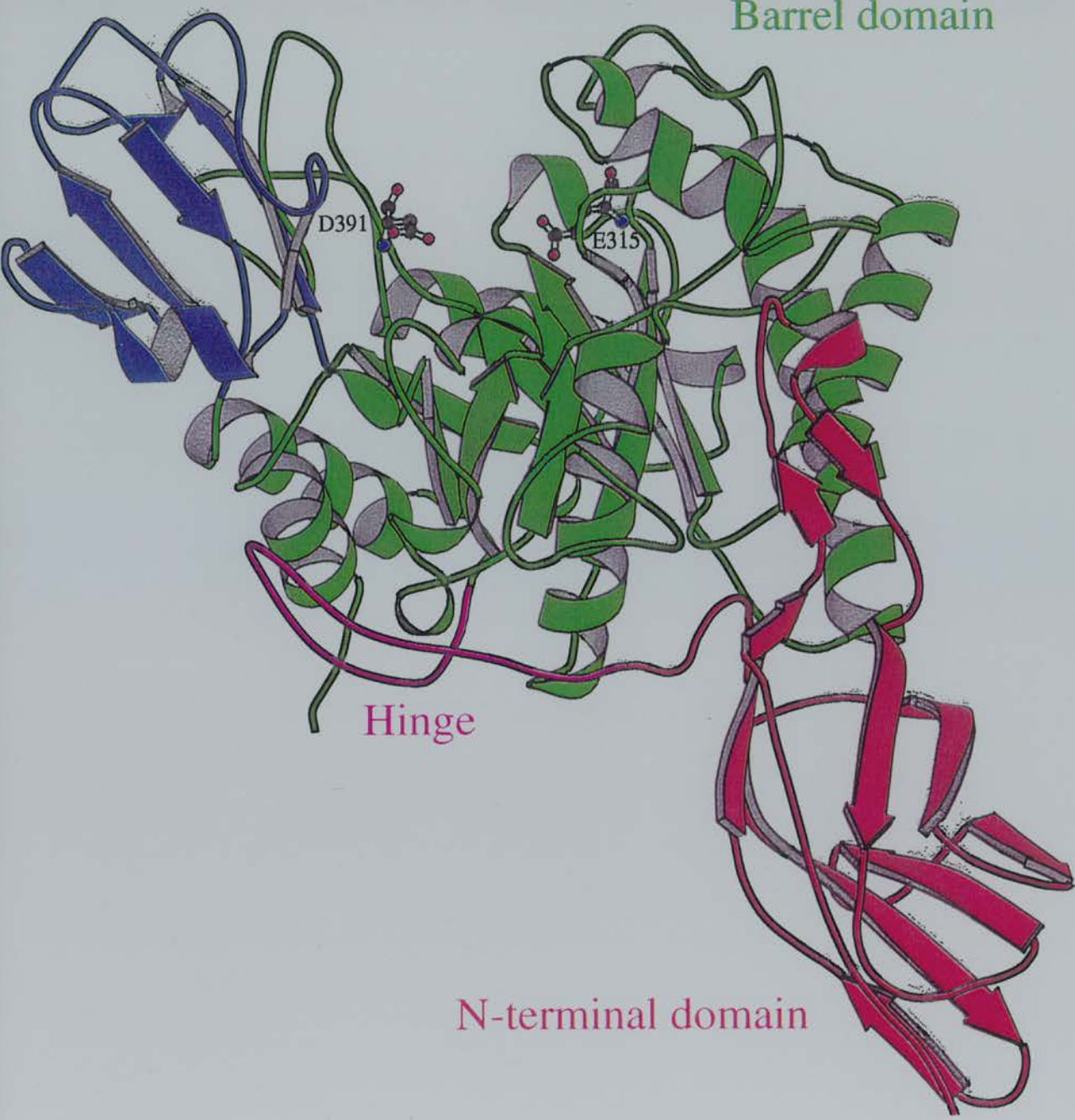


Figure 7.6 A ribbon structure of chitinase from *V. carchariae*

The model was built up by the program called "O" (Jones *et al.*, 1991). The *N*-terminal domain is represented in red, The hinge in magenta, the α/β -barrel domain in green, and a small domain in blue. Glu315 and Asp391 are presented in a ball and stick representation.

Small domain

Barrel domain



Hinge

N-terminal domain

displayed significant homology both in their catalytic cores and in their binding domains. In the *M. bispora* enzyme, the binding domain is at the C-terminus, while it is at the N-terminus in the *C. fimi*. Since both enzymes are endoglucanases, the location of the binding domains at the N- or C-terminus of the catalytic domain does not appear to determine the endo versus exo specificity of the enzymes.

The hinge region connects the amino-terminal domain to the rest of the structure. It is probably mobile and allows the amino-terminal domain to have different relative positions in solution.

The α/β -barrel domain is the catalytic domain of the enzyme. The active site is found to be located at the C-terminal part of the β -strands of the α/β -barrel, which is common in all known enzymes with α/β -barrel structure. The catalytic mechanism was proposed based on the *S. marcescens* chitinase structure in complex with N,N',N'',N''' tetra-acetylo-chitotetraose (tetraNAG) that the cleavage of chitin by ChiA was claimed to proceed by general acid-base catalysis, in a similar manner as in lysozyme and most glycosyl hydrolases. However, the most recent report (Brameld and Goddard, 1998b) proposed (based on molecular dynamic simulations) that the mechanism for *S. marcescens* and all other family 18 chitinases should follow the anchimeric stabilisation hydrolysis mechanism (see section 1.6.2, schematic 3).

The $\alpha+\beta$ fold domain is formed by an insertion between one β -strand and an α -helix in the α/β -barrel domain. However, functions of this domain still remain unknown.

7.8 Plan for Future Work

My plan for future work will be divided into three phases as follows:

The first phase : gene organisation

Since the gene encoding *V. carchariae* chitinase has been isolated, and expression of the chitinase in *E. coli* was preliminarily studied, it is possible to use the DNA sequence information to obtain more information about the genetic systems of chitinases in bacteria. For example, it would be interesting to know how many copies of chitinase genes are located in the genomic DNA of *V. carchariae*. DNA hybridisation techniques using DNA probes designed from highly conserved regions of the gene will be employed.

In addition, we now know that bacteria normally express more than one chitinase. It might be possible to use other DNA hybridisation probes to obtain some useful information about multiple gene systems in *Vibrio* and other bacteria that are classified as closely related species with *V. carchariae*, especially within the marine bacterial *Vibrio* group.

The second phase : expression

My work will also deal with trying to overcome the problems of very low enzyme activity detected in the current *E. coli* expression system, and to study about how the protein is processed. It was shown in this study that recombinant chitinase was expressed in *E. coli* XL1 Blue in relatively high amounts, but only a very small amount of chitinase activity was detected. We concluded that low chitinase activity was a result of the non-processed protein expressed by *E. coli*.

In addition, most of the protein produced was kept in the inclusion bodies, and thus might not be in a suitable conformation to be active. In the case of non-processed protein, we suspect that *E. coli* does not have the specific processing enzymes for *V. carchariae* chitinase. However, this problem might be overcome if we introduce the recombinant plasmid containing the chitinase gene into its wild-type species, and try to express the protein in a *Vibrio* system. We hope that the recombinant protein produced by *V. carchariae* will be processed, and produce the mature chitinase. As a

result of being processed, we also hope that the chitinase will be recovered in a fully active form.

Moreover, it has been shown that extracellular secretion of several proteins including chitinases in Gram-negative bacteria, especially as studied in *V. cholerae*, requires the general secretion pathway and a cluster of *eps* genes appears to take part in this respect (as discussed in section 6.7.6). Probably, the current *E. coli* expression system does not contain the specific *eps* genes that are responsible for chitinase secretion. If they are present, the gene system might not be expressed properly (Sandkvist *et al.*, 1997) or function fully for protein secretion as discussed previously in case of cholera toxin (CT) and heat-labile enterotoxin (LT). After the signal peptides of the A and B precursor polypeptides are cleaved off during export as they cross the cytoplasmic membrane, assembly of the subunits occurs in the periplasm. When assembled, both CT and LT are secreted from *V. cholerae*, but in *E. coli*, they remain in the periplasm (Connell *et al.*, 1998). If this is the case, expression in other secretion systems will be considered. However, expression in the *Vibrio* system might be an advantage as at least it has been indicated that the *eps* genes exist in the *Vibrio* system. It is hoped that the recombinant chitinase would be expressed and secreted extracellularly as a fully functional enzyme.

In the case of the protein in inclusion bodies, optimisation of conditions for growing cells i.e. growing time, pH of the growth media, temperature for growing cells and concentration of chitin added in the medium, will be tried to increase the yields of soluble chitinase. Development of a refolding scheme for the inclusion body protein might also be considered. Optimisation of refolding conditions to obtain active enzyme will be tried in this case. To try to obtain active enzyme from inclusion bodies might be a useful strategy as it has been suggested that the starting protein material will be substantially purified chitinase, and that therefore the development of a purification scheme might not be difficult to achieve.

My work during the second phase will also deal with development of a high expression system of the chitinase for detailed characterisation and for molecular

structure studies. Even though the current *E. coli* expression system gave a relatively high quantity of chitinase, the chitinase gene might need to be introduced into a more suitable bacterial expression system either to obtain higher yields of the enzyme or to obtain secreted enzyme for bioconversion purposes. Alternatively, site-directed mutagenesis using PCR techniques might be employed to modify the molecule of the chitinase to obtain the higher chitinase expression in *E. coli*. For example, some particular nucleotides at the control region of the gene might be mutated in order to obtain consecutive gene expression.

In addition, apart from site-directed mutagenesis techniques, enzyme kinetics will also be used to study the mode of enzyme action. For example, the importance of the conserved amino acid residues, i.e. Glu315, and Asp391, and other residues that are suggested to play important roles in the enzyme catalysis, will be verified.

Moreover, site-directed mutagenesis might be used to modify the molecule of the enzyme to obtain a chitinase with some improved properties, i.e. stable at a broad range of temperature or organic solvents, that will be suitable under fermentation conditions, such as used by Carroad (Cosio *et al.*, 1982) for treatment of chitin waste.

The third phase : structural studies and biotechnology applications

My work during this phase will be to accomplish the long-term aim of the project. Once a high expression system has been established, crystals of the enzyme will be prepared in order to determine the three dimensional structure of the enzyme. We hope that the crystal structure of the enzyme will help us to understand more the relationships between structure and enzyme function. Even though the mechanism of the family 18 chitinase enzyme has been proposed based on molecular dynamic simulations using the known structure of *S. marcescens* as a model, it would still be important to verify the proposed mechanism using the crystal structure of other chitinases in the same family. Moreover, once the structure of the *V. carchariae* is obtained, it might help expand our knowledge to understand the roles of other

domains, the *N*-terminal domain, and the $\alpha+\beta$ small domain, in the enzyme molecule.

Development of a fermentor culture system for recycling chitin waste in the seafood industry will be included in this phase. Conditions for enzyme production and for chitin degradation i.e. controlled temperature and pH in the culture fermentor and concentration of chitin for chitinase induction, to obtain high yields of the chitin products will need to be optimised.

Appendix I

References

- Alam, M.M., Nikaidon, N., Tanaka, H., and Watanabe, T.C., 1995. Cloning and sequencing of ChiC gene of *Bacillus circulans* WL-12 and relationship of its product to some other chitinases and chitinase like proteins. *J. Ferment. Bioeng.* 80, 454-461
- Anke, C., van Scheltinga, T., Hennig, M., and Dijkstra, B.W., 1996. The 1.8 Å resolution structure of hevamine, a plant chitinase/lysozyme, and analysis of the conserved sequence and structure motifs of glycosyl hydrolases family 18. *J. Mol. Biol.* 262, 243-257
- Audi, P., Quere, D.L., Leclerc, D., and Asselin, A., 1990. Electrophoretic forms of lysozyme activity in various plant species. *Phytochemistry* 29, 1143-1159
- Ausubel, F.M., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struh, L.K., 1994. Preparation and analysis of DNA. In *Current Protocols in Molecular Biology*. (Ausubel, F.M., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struh, L.K., ed.). Sections 2.4.1-2.4.5 (2), John Wiley & Son. Inc., U.S.A
- Bassler, B.L., Gibbons, P.J., Yu, C., and Roseman, S., 1991a. Chitin utilization by marine bacteria. Chemotaxis to chitin oligosaccharides by *Vibrio furnissii*. *J. Biol. Chem.* 266, 2468-2475
- Bassler, B.L., Yu, C., Lee, Y.C., and Roseman, S., 1991b. Chitin utilization by marine bacteria. Degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. *J. Biol. Chem.* 266, 2476-2486
- Bèguin, P., and Aubert, J.P., 1994. The biological degradation of cellulose. *FEMS Microbiol. Rev.* 13, 25-58
- Blackwell, J., 1988. Physical methods for the determination of chitin structure and conformation. *Methods Enzymol.* 161, 435-442

Boller, T., Gehri, A., Mauch, F., and Vogeli, U., 1983. Chitinase in bean leaves: induction by ethylene, purification, properties, and possible function. *Planta (Berl.)* 157, 22-31

Boller, T., and Metraux, J.P., 1988. Extracellular localization of chitinase in cucumber. *Physiol. Mol. Plant Pathol.* 33, 11-16

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72, 248-254

Brameld, K.A., and Goddard III, W.A., 1998a. The role of enzyme distortion in the single displacement mechanism of family 19 chitinases. *Proc. Nat. Acad. Sci.* 95, 4276-4281

Brameld, K.A., and Goddard III, W.A., 1998b. Substrate distortion to a boat conformation at subsite -1 is critical in the mechanism of family 18 chitinases. *J. Am. Chem. Soc.* 120, 3571-3580

Brine, C.J., 1984. Chitin: Accomplishments and Perspectives. In Chitin, Chitosan, and Related enzymes (Zakikas, J.P., ed.), xxii-xxiv, Academic Press, New York

Cabib, E., 1987. The synthesis and degradation of chitin. *Adv. Enzymol. Related Areas Mol. Biol.* 59-101

Cabib, E., 1988. Chitin. *Methods Enzymol.* 161, 425-435

Carroad, P.A., and Tom, R.A., 1978. Bioconversion of shellfish chitin wastes: process conception and selection of microorganisms. *J. Food. Sci.* 43, 1158-1161

Cherif, M., and Benhamou, N., 1990. Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. *radicislycopersici*. *Phytopathology* 80, 1406-1414

Chernin, L.S., de la Fuente, L., Sobolev, V., Haran, S., Vorgias, C., Oppenheim, A.B., and Chet, I., 1997. Molecular Cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. *Appl. Environ. Microbiol.* 63(3), 834-839

Chet, I., 1987. *Trichoderma* application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*. (Chet, I., ed.). 137-160, John Wiley and Sons, *New York*

Clark, J., Quayle, K.A., Macdonald, N.L., and Stark, J.R., 1988. Metabolism in marine flatfish -V. chitinolytic activities in dover sole, *Solea Solea* (L.). *Comp. Biochem. Physiol.* 90B(2), 379-384

Connell, T.D., Metzger, D.J., Lynch, J., and Folster, J.P., 1998. Endochitinase is transported to the extracellular milieu by the *eps*-encoded general secretory pathway of *Vibrio cholerae*. *J. Bacteriol.* 180(21), 5591-5600

Cosio, I.G., Fisher, R.A., and Carroad, P.A., 1982. Bioconversion of shellfish chitin waste: waste pretreatment, enzyme production, process design, and economic analysis. *J. Food Sci.* 47, 901-905

Coutinho, J.B., Gilkes, N.R., Kilburn, D.G., Warren, R.A.J., and Miller, R.C., 1993. The nature of the cellulose-binding domain affects the activities of a bacterial endoglucanase on different forms of cellulose. *FEMS Microbiol. Lett.* 113, 211-218

Cronin, D., Moënné-Loccoz, Y., Dunne, C., and O'Gara, F., 1997. Inhibition of egg hatch of the potato cyst nematode *Globodera rostochiensis* by chitinase-producing bacteria. *Eur. J. Plant. Pathology* 103, 433-440

- Davies, G., and Henrissat, B., 1995. Structures and mechanism of glycosyl hydrolases. *Structure* 3, 853-859
- Davis, B., and Eveleigh, D.E., 1984. Chitosanases: Occurrence, Production, and Immobilization. In *Chitin, Chitosan, and Related Enzymes* (Zakikas, J.P., ed.). 160-179. Academic Press, *New York*
- de Jong, A.J., Cordewener, J., lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A., de Vries, S.C., 1992. A carrot somatic embryo mutant is rescued by chitinase. *The Plant Cell* 4, 425-433
- d'Enfert, C., Ryter, A., and Pugsley, A.P., 1987. Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. *EMBO J.* 6, 3531-3538
- Elad, Y., Chet, I., and Henis, Y., 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. *Can. J. Microbiol.* 28, 719-725
- Fry, S.C., 1988. Separation and identification of degradation products. In *The growing plant cell wall: chemical and metabolic analysis*. (Fry, S.C., ed.). 153-179, John Wiley & Sons, Inc., *New York*
- Fuchs, R.L., Mcpherson, S.A., and Drahos, D.J., 1986. Cloning of a *Serratia marcescens* gene encoding chitinase. *Appl. Environ. Microbiol.* 51, 504-509
- Fukamizo, T., Koga, D., and Goto, S., 1995. Comparative Biochemistry of chitinases -anomeric form of the reaction products. *Biosci. Biotechnol. Biochem.* 59, 311-313
- Fukasawa, S., Arai, M., Wada, T., Shima, H., and Kurata, M., 1992. Some properties of a chitinase from a marine luminous bacterium, *Vibrio fischeri* strain COT-A136. *Chem. Pharm. Bul.* 40, 1631-1633

Gal, S.W., Choi, J.Y., Kim, C.H., Cheong, Y.H., Choi, Y.J, Lee, S.Y., Bahk, J.D., and Cho, M.J., 1998. Cloning of the 52-kDa chitinase gene from *Serraria marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme. *FEMS Microbiol. Lett.* 160, 151-158

Georgopapadakou, N.H., and Tkacz, J.S., 1995. The fungal cell wall as a drug target. *Trends Microbiol.* 3, 98-104

Ghigo, J.-M., and Wandersman, C., 1992. Cloning, nucleotide sequence and characterization of the gene encoding the *Erwinia chrysanthemi* B374 PrtA metalloprotease: a third metalloprotease secreted via a C-terminal secretion signal. *Mol. Gen. Genet.* 236, 135-144

Gilkes, N.R., Claeyssens, M., Aebersold, R., Henrissat, B., Meinke, A., Morrison, H.D., Kilburn, D.G., Warren, R.A.J., and Miller, R.C., 1991a. Structural and functional relationships in two families of β -1,4-glycanases. *Europ. J. Biochem.* 202, 367-377

Gilkes, N.R., Henrissat, B., Kilburn, D.G., Miller, R.C., and Warren, R.A.J., 1991b. Domains in microbial β -1,4-glucanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55, 303-315

Gilkes, N.R., Kilburn, D.G., Miller, R.C., and Warren, R.A.J., 1989. Structural and functional analysis of a bacterial cellulase by proteolysis. *J. Biol. Chem.* 264, 17802-17808

Hackman, R.H., and Golberg, M., 1964. New substrates for use with chitinases. *Anal. Biochem.*, 397-401

Hames, B.D., 1990. One-dimensional polyacrylamide gel electrophoresis. In Gel electrophoresis of proteins: a practical approach. (Hames, B.D., and Rickwood, D., 2nd ed.). 528-544, Oxford University Press, England

- Harpster, M.H., and Dunsmuir, P., 1989. Nucleotide sequence of the chitinase B gene of *S. marcescens* QMB 1466. *Nucleic Acid Res.* 17, 5395
- Hedrick, S.A., Bell, J.N., Boller, T., and Lamb, C.J., 1988. Chitinase cDNA cloning and mRNA induction by fungal elicitor, wounding, and infection. *Plant. Physiol.* 86, 182-186
- Henrissat, B., 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280, 309-316
- Henrissat, B., and Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarity. *Biochem. J.* 316, 695-696
- Henrissat, B., and Bairoch, A., 1996. Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316, 695-696
- Hiraga, K., Shou, L., Kitazawa, M., Takahashi, S., and Shimada, M., Sato, R., and Oda, K., 1997. Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330. *Biosci. Biotech. Biochem.* 61(1), 174-176
- Hirono, I., Yamashita, M., and Aoki, T., 1998. Note: Molecular cloning of chitinase genes from *Vibrio anguillarum* and *V. parahaemolyticus*. *J. Appl. Microbiol.* 84, 1175-1178
- Hirst, T.R., Hardy, S.J.S., Randall, L.L., 1983. Assembly in vivo of enterotoxin from *Escherichia coli*: formation of the B subunit oligomer. *J. Bacteriol.* 153, 21-26
- Hofstra, H., and Witholt, B., 1984. Kinetics of synthesis, processing, and membrane transport of heat-labile enterotoxin, a periplasmic protein in *Escherichia coli*. *J. Biol. Chem.* 259, 15182-15187

Hofstra H., and Witholt, B., 1985. Heat-labile enterotoxin in *Escherichia coli*. Kinetics of association of subunits into periplasmic holotoxin. *J. Biol. Chem.* 260, 16037-16044

Holland, I.B., Blight, M.A., and Kenny, B., 1990. The mechanism of secretion of hemolysin and other polypeptides from Gram-negative bacteria. *J. Bioenerg. Biomemb.* 22, 473-491

Howitz, M., Reid, J.D., and Ogrydziak, D., 1984. Genetic improvement of chitinase production by *Serratia marcescens*. In Chitin, Chitosan, and Related Enzyme (Zakikas, J.P., ed.) 190-208, Academic Press, New York

Huang, J-H., Chen, C-J., and Su Y-C., 1996. Production of chitinolytic enzymes from a novel species of *Aeromonas*. *J. Industrial. Microbiol.* 17, 89-95

Imoto, T., and Yagishita, K., 1971. A simple activity measurement of lysozyme. *Agric. Biol. Chem.* 35, 1154-1156

Inbar, J., and Chet, I., 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by this bacterium. *Soil Biol. Biochem.* 23, 973-978

Iseli, B., Armand, S., Boller, T., Neuhaus, J-M., Henrissat, B., 1996. Plant chitinases use two different hydrolytic mechanisms. *FEBS Lett.* 382, 186-188

Ishimaru, K., Akagawa-Matsushita, M., and Muroga, K., 1996. *Vibrio ichthyenteri* sp. nov., a pathogen of Japanese flounder (*Paralichthys olivaceus*) larvae. *Int. J. Sys. Bacteriol.* 46(1), 155-159

Jannatipour, M., Soto-Gil, W.R., Childers, L.C., and Zyskind, J.W., 1987. Translocation of *Vibrio harveyi* N,N'-Diacetylchitobiase to the outer membrane of *Escherichia coli*. *J. Bacteriol.* 169(8), 3785-3791

- Jeuniaux C., 1961. Chitinase: an addition to the list of hydrolases in the digestive tract of vertebrates. *Nature, Lond.* 192, 135-136
- Jeuniaux C. 1966. Chitinases. *Methods Enzymol.* 8, 644-650
- Jones, J.D.G., Grady, K.L., Suslow, T.V., and Bedbrook, J.R., 1986. Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *EMBO J.* 5, 467-473
- Jones, T.A., Zou, J-Y., Cowan, S.W., and Kjeldgaard, M., 1991. Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Cryst.* A47, 110-119
- Julio, C., Villagómez-Castro, and López-Romero, E., 1996. Identification and partial characterization of three chitinase forms in *Entamoeba invadens* with emphasis on their inhibition by allosamidin. *Antonie van Leeuwenhoek* 70, 41-48
- Kaiser, K., Murray, N.E., and Whittaker, P.A., 1996. Construction of representative genomic DNA libraries using phage lambda replacement vectors. In *DNA Cloning 1: A Practical Approach*. (Glover, D.M., and Hames B.D., 2nd ed.). 37-84, Oxford University Press Inc., *New York*
- Kargh, K.M., Jacobsen, S., Mikkelsen, J.D., and Nielsen, K.A., 1991. Purification and characterization of three chitinases and one β -1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). *Plant Science* 76, 65-77
- Keefe, D., Hinz, U., and Meins, F., 1990. The effect of ethylene on the cell: Type-specific and intracellular localization of (1-3) β -glucanase and chitinase in tobacco leaves. *Planta (Berl.)* 182, 43-51

- Kuranda, M., and Robbins, P.W., 1991, Chitinase is required for cell separation during growth of *Saccharomyces cerevisiae*. *J. Biol. Chem.* 266, 19758-19767
- Kurosaki, F., Tashiro, N., and Nishi, A., 1987. Induction, purification and possible function of chitinase in cultured carrot cells. *Physiol. Mol. Plant Pathol.* 31, 201-210
- Kurosaki, F., Tashiro, N., and Nishi, A., 1988. Role of chitinase and chitin oligosaccharides in lignification response of cultured carrot cells treated with mycelial walls. *Plant Cell Physiol.* 29, 527-531
- Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of head of bacteriophage -T₄. *Nature* 227, 680-685
- Laine, R.A., Ou, C., Lo, J., and Fossett, M.C., 1988. Molecular cloning, expression, purification, and characterization of a chitinase from *Vibrio parahaemolyticus*. *Glycoconjugate J.* 5, 367
- Lever, M., 1972. A new reaction for colorimetric determination of carbohydrates. *Anal. Biochem.* 47, 273-279
- López-Romero, E., and Villagómez-Castro, J.C., 1993. Encystation in *Entamoeba invadens*. *Parasitology Today* 9, 225-227
- Lorito, M., Pietro, A., Hayes, C.K., Woo, S.L., and Harman, G.E., 1993. Antifungal, synergistic interaction between chitinolytic enzymes from *Trichoderma harzianum* and *Enterobacter cloacae*. *Phytopathology.* 83, 721-728
- Lorito, M., Woo, S.L., Fernandez, I.G., Colucci, G., and Harman, G.E., 1998. Genes from mycoparasitic fungi as a source for improving plant resistance to fungal pathogens. *Proc. Natl. Acad. Sci. USA.* 95(14), 7860-7865

- Maniatis, T., Fritsch, E.F., and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory.
- Mauch, F., Hadwiger, L.A., and Boller, T., 1984. Ethylene symptom, no signal for the induction of chitinase and β -1,3-glucanase in pea pods by pathogens and elicitors. *Plant. Physiol. (Bethesda)* 76, 607-611
- Mauch, F., and Staehelin, L.A., 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and β -1,3-glucanase in bean leaves. *Plant Cell* 1, 447-457
- Manocha, M.S., and Zhonghua, Z., 1997. Immunocytochemical and cytochemical localization of chitinase and chitin in the infected hosts of a biotrophic mycoparasite, *Piptocephalis virginiana*. *Mycologia* 89(2), 185-194
- Manuel, A., S., Tuite, S., and Tuite, M.F., 1993. New insights into mRNA decoding-implications for heterogenous protein synthesis. *TIBTECH.* 11, 500-505
- McClure, W.R., 1985. Mechanism and control of transcription initiation in prokaryotes. *Ann. Rev. Biochem.* 54, 171-204
- Miyashita, K., Fujii, T., and Sawada, Y., 1991. Molecular cloning and characterization of chitinase genes from *Streptomyces lividans* 66. *J. Gen. Microbiol.* 137, 2065-2072
- Molano, J., Polacheck, I., Duran, A., and Cabib, E., 1979. An endochitinase from wheat germ. Activity on nascent and preformed chitin. *J. Biol. Chem.* 254, 4901-4907
- Monreal, J., and Reese, E.T., 1968. The chitinase of *Serratia marcescens*. *Can. J. Microbiol.* 15, 689-696

Montgomery, M.T., and Kirchman, D.L., 1993. Role of chitin-binding proteins in the specific attachment of the marine bacterium *Vibrio harveyi* to chitin. *App. Environ. Microbiol.* 59(2), 373-379

Murao, S., Kawada, T., Itoh, H., Oyama, Hiroshi., and Shin, T., 1992. Purification and characterization of a novel type of chitinase from *Vibrio alginolyticus* TK-22. *Biosci. Biotech. Biochem.* 56(2), 368-369

Ohishi, K., Ishikawa, F., and Nomoto, M., 1989. Chitinolytic and lysozyme activities in plants. In Chitin and chitosan. (Braek, G.S., Anthonsen, T., and Sanford, P., ed.). 185-195, Elsevier Science Publishers Ltd., *Amsterdam*

Okutani, K., 1967. Studies of chitinolytic systems in the digestive tract of *Latelabrax japonicus*. *Bull. Misaki Mar. Biol. Inst.* 10, 1-47

Okutani, K., Sawada, T., and Kimata, M., 1967. Studies of chitinolytic enzymes in aquatic animals -VI. The chitinolytic enzyme present in rainbow trout. *Bull. Jap. Soc. Sci. Fish.* 33, 952-955

Okutani, K., 1977. Chitin digestion in the digestive tract of fish. *Proc. First Int. Confer. Chitin/Chitosan*, Boston MA (USA), 11-13, April, 1977, 554-562

Osawa, R., and Koga, T., 1995. An investigation of aquatic bacteria capable of utilising chitin as the sole source of nutrients. *Lett. Appl. Microbiol.* 21, 288-291

Overbye, L.J., Sandkvist, M., and Bagdasarian, M., 1993. Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene*. 132, 101-106

Papavizas, C.G., 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. *Ann. Rev. Phytopathol.* 23, 23-54

Pedesen, K., Verdonck, L., Austin, B., Austin, D.A., Blanch, A.R., Grimont, P.A.D., Jofre, J., Koblavi, S., Larsen, J.L., Tiainen, T., Vigneulle, M., and Swings, J., 1998. Taxonomic evidence that *Vibrio carchariae* Grimes *et al.* 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann *et al.* 1981. *Int. J. Sys. Bacteriol.* 48, 749-758

Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., Wilson, K.S., and Vorgias, C.E., 1994. Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure* 2, 1169-1180

Perrakis, A., Ouzounis, C., and Wilson, K.S., 1997. Evolution of immunoglobulin-like modules in chitnases: their structural flexibility and functional implications. *Folding & Design* 2(5), 291-294

Pleban, S., Chernin, L., and Chet, I., 1997. Chitinolytic activity of an endophytic strain of *Bacillus cereus*. 25, 284-288

Rao, V.H., Guan, C., and van Roey, P., 1995. Crystal structure of endo- β -N-acetylglucosaminidases H at 1.9 Å resolution: active-site geometry and substrate recognition. *Structure* 3, 449-457

Reid, J.D., and Ogrydziak, D.M., 1981. Chitinase-overproducing mutant of *Serratia marcescens*. *Appl. Environ. Microbiol.* 41, 664-669

Ridout, C.J., Coley-Smith, J.R., Lynch, J.M., 1986. Enzyme activity and electrophoretic profile of extracellular protein are induced in *Trichoderma spp.* by cell walls of *Rhizoctonia solani*. *J. Gen. Microbiol.* 132, 2345-2352

Sakuda, S., Isogai, A., Matsumoto, S., and Suzuki, A., 1986. The structure of allosamidin, a novel insect chitinase inhibitor, produced by *Streptomyces sp.* *Tetrahedron Lett.* 27, 2475-2478

Sandkvist, M., Michel, L.O., Hough, L.P., Morales, V.M., Bagdasarian, M., Koomey, M., Dirita, V.J., and Bagdasarian, M., 1997. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* 179(22), 6994-7003

Sedgwick, S.G., thi Man, N., Ellis, J.M., Crowne, H., and Morris, G.E., 1991. Rapid mapping by transposon mutagenesis of epitopes on the muscular dystrophy protein, dystrophin. *Nucleic Acids Research* 19(21), 5889-5894

Shahabuddin, M., and Kaslow, D.C., 1993. Chitinase: a novel target for blocking parasite transmission? *Parasitology Today* 9, 252-255

Shapiro, R., Ordentlich, A., Chet, I., and Oppenheim, A.B., 1989. Control of plant diseases by chitinase expressed from cloned DNA in *Escherichia coli*. *Phytopathology* 79, 1246-1249

Shigemasa, Y., Saito, K., Sashiwa, H., and Saimoto, H., 1994. Enzymatic degradation of chitins and partially deacetylated chitins. *Int. J. Biol. Macromol.* 16, 43-49

Shin, W.C., Lee, D.S., Kim, T.H., Woo, J.H., Lee, J.M., Kim, J.G., and Hong, S.D., 1995. Isolation and characterization of *Acinetobacter* sp. WC-17 producing chitinase. *J. Microbiol. Biotechnol.* 5, 80-86

Shin, W.C., Lee, D.S., Kim, J.G., and Hong, S.D., 1995. Molecular cloning of *Acinetobacter* sp. WC-17 gene encoding chitinase. *J. Microbiol. Biotechnol.* 5, 87-91

Shinshi, H., Neuhaus, J.-M., Ryals, J., and Meins, F. Jr., 1990. Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cysteine-rich domain. *Plant Mol. Biol.* 14, 357-368

- Sivan, A., and Chet, I., 1989. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. *J. Gen. Microbiol.* 135, 675-682
- Soto-Gil, R.W., and Zyskind, J.W., 1984. Cloning of *Vibrio harveyi* chitinase and chitobiase genes in *Escherichia coli*. In *Chitin, Chitosan, and Related Enzymes* (Zakikas, J.P., ed.). 209-233, Academic Press, *New York*
- Soto-Gil, R.W., 1988. Chitobiase and chitinase from *V. harveyi*. PhD dissertation, University of California, San Diego and San Diego State University, *California*
- Splindler, K.D., Splindler-Barth, M., and Londershausen, M., 1990. Chitin metabolism: a target for drugs against parasites. *Parasitol. Res.* 76, 283-288
- Spindler-Barth, M., 1993. Hormonal regulation of chitin metabolism in insect cell lines. In *Chitin Enzymology* (Muzzarelli, R.A.A., ed.) 75-82, European Chitin Society, *Italy*
- Srivastava, A.K., Defago, G., and Boller, T., 1985. Secretion of chitinase by *Aphanocladium album*, a hyperparasite of wheat rust. *Experientia*. 41, 1612-1613
- Stryer, L., 1995. Protein targeting. In *Biochemistry*. (Stryer, L., 4th ed.). 911-948, W.H. Freeman and Company, *New York*
- Svensson, B., Jespersen, H., Sierks, M.R., and Macgregor, E.A., 1989. Sequence homology between putative raw-starch binding domains from different starch degrading enzymes. *Biochem. J.* 264, 309-311
- Svityl, A.L., Chadhain, S.M.N., Moore, J.A., and Kirchman, D.L., 1997. Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* growing on different forms of chitin. *Appl. Environ. Microbiol.* 63, 408-413

Svityl, A.L., and Kirchman, D.L., 1998. A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases: implications for the ecology and evolution of 1,4- β -glycanases. *Microbiology* 144, 1299-1308

Takahashi, M., Tsukiyama, T., and Suzuki, T., 1993. Purification and some properties of chitinase produced by *Vibrio* sp. *J. Ferment. Bioeng.* 75(6), 457-459

Thompson, J.D., Higgins, D.G., and Gibson, T.J., 1994. CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673-4680

Trachuk, L.A., Shemyakina, T.M., Chestukhina, G.G., and Stepanov, V.M., 1996. Chitinases from *Bacillus cereus*: isolation and characteristics. *Biochemistry (Moscow)* 61(2), 267-275

Tsujibo, H., Yoshida, Y., and Miyamoto, K., 1992. Purification, properties, and partial amino acid sequences of chitinase from a marine *Alteromonas* sp. strain O-7. *Can. J. Microbiol.* 38, 891-897

Tsujibo, H., Orikoshi, H., Tanno, H., Fujimoto, K., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y., 1993. Cloning, sequence, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7. *J. Bacteriol.* 175, 176-181.

Tsujibo, H., Orikoshi, H., Shiotani, K., Hayashi, M., Umeda, J., Miyamoto, K., Imada, C., Okami, Y., and Inamori, Y., 1998. Characterization of chitinase C from a marine bacterium, *Alteromonas* sp. strain O-7, and its corresponding gene and domain structure. *App. Environ. Microbiol.* 64(2), 472-478

Ueda, M., Kawaguchi, T., and Arai, M., 1994. Molecular cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas* sp. NO. 10S-24. *J. Ferment. Biotechnol.* 78, 205-211

van Roey, P., Rao, V., Plummer, T.H.Jr., and Tarentino, A.L., 1994. Crystal structure of endo- β -*N*-acetylglucosaminidases F₁, an α/β -barrel enzyme adapted for a complex substrate. *Biochemistry* 33, 13989-13996

van Scheltinga, T., A.C., Kalk, K.H., Beintema, J.J., Dijkstra, B.W., 1994. Crystal structures of hevamine, a plant defense protein with chitinase and lysozyme activity, and its complex with inhibitor. *Structure* 2, 1181-1189.

von Heijne, G., 1983. Patterns of amino acids near single sequence cleavage sites. *Eur. J. Biochem.* 133, 17-21

Vorgias, C.E., Perrakis, A., and Tews, I., 1996. Structure-function studies on the chitinolytic enzymes of *Serratia marcescens* chitinase and chitobiase. *Ann. New York Acad. Sci.* 190-192

Wang, S-L., and Chang, W-T., 1997. Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium. *Appl. Environ. Microbiol.* 63(2), 380-386

Wang, S.H., Zheng, H.J., Dissanayake, S., Cheng, W.F., Tao, Z.H., Lin, S.Z., Piessens, W.F., 1997. Evaluation of recombinant chitinase and SXP1 antigens as antimicrofilarial vaccines. *Am. J. Trop. Med. Hyg.* 56(4), 474-481

Ward, K., and Seib, P.A., 1970. Chitin. In *The Carbohydrates; Chemistry and Biochemistry*. (Pigman, W., and Horton, D., 2nd ed.). 435-439, Academic Press, *New York and London*

Watanabe, T., Oyanagi, W., Suzyki K., and Tanaka, H., 1990. Chitinase system of *Bacillus circulans* WL-12 and importance of chitinase A1 in chitin degradation. *J. Bacteriol.* 172,4107-4022

Watanabe, T., Oyanagi, W., Suzuki, K., Ohnishi, K., and Tanaka, H., 1992. Structure of the gene encoding chitinase of *Bacillus circulans* WL-12 and possible homology of the enzyme to other prokaryotic chitinases and class III plant chitinases. *J. Bacteriol.* 174, 408-414

Watanabe, T., Kobori, K., Mayashita, K., Fujii, T., Sakai, H., Uchida, M., and Tanaka, H., 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 268(25), 18567-18572

Watanabe, T., Kimura, K., Sumiya, T., Nikaidou, N., Suzuki, K., Suzuki, M., Taiyoji, M., Ferrer, S., and Regue, M., 1997. Genetic analysis of the chitinase system of *Serratia marcescens* 2170. *J. Bacteriol.* 179(22), 7111-7117

Wiwat, C., Lertcanawanichakul, M., and Siwayapram, P., 1996. Expression of chitinase-encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *israelensis*. *Gene* 179, 119-126

Wortman, A.T., Somerville, C.C., and Colwell, P.R. 1986. Chitinase determinants of *Vibrio vulnificus*: gene cloning and applications of a chitinase probe. *Appl. Environ. Microbiol.* 52, 142-145

Woo, C-J, Yun, U-J., and Park, H-D., 1996. Isolation of chitin-utilizing bacterium and production of its extracellular chitinase. *J. Microbiol. Biotechnol.* 6(6), 439-444

Yamamoto, Y., Fukunaga, Y., Aoyagi, H., and Tanaka, H., 1995. Purification and characteristics of chitinase secreted by cultured *Wasabia japonica* cells. *J. Ferment. Bioeng.* 80(2), 148-152

Yu, C., Lee, A.M., Bassler, B.L., and Roseman, S., 1991. Chitin utilization by marine bacteria. A physical function for bacterial adhesion to immobilized carbohydrates. *J. Biol. Chem.* 266, 24260-24267

Appendix II

Bacterial Media

1 Bacterial media

1.1 LB Medium (Luria-Bacterial Medium)

Per litre:

To 950 ml of distilled H₂O add:

bacto-tryptone 10 g

bacto-yeast extract 5 g

NaCl 10 g

Stir until the solutes have dissolved. Adjust the volume of the solution to 1 litre with distilled H₂O. Sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.

1.2 SOB Medium

Per litre:

To 950 ml of distilled H₂O, add:

bacto-tryptone 20 g

bacto-yeast extract 5 g

NaCl 0.5 g

Stir until solutes have dissolved. Add 10 ml of a 250 mM solution of KCl. (This solution is made by dissolving 1.86 g of KCl in 100 ml of distilled H₂O). Adjust the pH to 7.0 with 5 N NaOH (~2.0 ml). Adjust the volume of the solution to 1 litre with distilled H₂O. Sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.

Just before use, add 5 ml of a sterile solution of 2 M MgCl₂. (This solution is made by dissolving 19 g of MgCl₂ in a 90 ml of distilled H₂O. Adjust the volume of the solution to 100 ml with distilled H₂O, and sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.)

1.3 SOC medium

SOC medium is identical to SOB medium, except that it contains 20 mM glucose. After the SOB medium has been autoclaved, allow it to cool to 60°C or less and then add 20 ml of a sterile 1 M solution of glucose. (This solution is made by dissolving 18 g of glucose in 90 ml of distilled H₂O. After the sugar has dissolved, adjust the volume of the solution to 100 ml with distilled H₂O and sterilise by filtration through a 0.22-micron filter.)

1.4 VCM (Vibrio Complex Medium), pH 8.5

Per litre:

To 950 ml of distilled H₂O add:

Bacteriological peptone	5 g
bacto-yeast extract	5 g
NaCl	30 g

Stir until solutes have dissolved. Tris.HCl was added to the medium to a final concentration of 50 mM. Adjust the pH to 8.5 with 5 N NaOH. Adjust the volume of the solution to 1 litre with distilled H₂O. Sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.

1.5 Marine Medium 2216E

Per litre:

To 950 ml of filtered, aged sea water add:

Bacteriological peptone	5 g
FePO ₄	0.10 g
bacto-yeast extract	5 g

Stir until solutes have dissolved. Adjust the pH to 7.5-7.6 with 1 N NaOH. Adjust the volume of the solution to 1 litre with filtered, aged sea water. Sterilise by autoclaving for 20 min at 15lb/sq. in. on liquid cycle.

1.6 Media Containing Agar

Prepare liquid media according to the recipe given above. Just before autoclaving, add bacto-agar (15g/litre), then sterilise by autoclaving for 20 min at 15 lb. in. on liquid cycle. When the medium is removed from the autoclave, swirl it gently to distribute the melted agar evenly throughout the solution. The medium is allowed to cool to 50°C before adding thermolabile substances (e.g., antibiotics). To avoid producing air bubbles, mix the medium by swirling. Plates are then poured directly from the flasks; allow about 30-35 ml of medium per 90-mm plate. To remove bubbles from the medium in the plate, the surface of the medium is flamed with bunsen burner before the agar hardens.

1.7 Chitin Containing Media

Prepare liquid or agar media to the recipe given above, except that 1% (w/v) or 5% (w/v) sulphuric acid-treated chitin (swollen chitin) is added before adjusting pH and autoclaving.

Appendix III

Solutions for Protein Studies

1. SDS-PAGE

1.1 Solutions for Preparing Resolving SDS-Polyacrylamide Gels

Solution components	5 ml	<u>Component volume (ml)</u>	
		10 ml	20 ml
10%			
H ₂ O	1.9	4.0	7.9
30% acrylamide mix*	1.7	3.3	6.7
1.5 M Tris (pH8.8)	1.3	2.5	5.0
10% SDS	0.05	0.1	0.2
10% ammonium persulphate	0.05	0.1	0.2
TEMED	0.002	0.004	0.008
12%			
H ₂ O	1.6	3.3	6.6
30% acrylamide mix	2.0	4.0	8.0
1.5 M Tris (pH8.8)	1.3	2.5	5.0
10% SDS	0.05	0.1	0.2
10% ammonium persulphate	0.05	0.1	0.2
TEMED	0.002	0.004	0.008

* 30% acrylamide mix consists of 30% (w/v) acrylamide and 0.8% (w/v) *N,N'*-methylene-bis-acrylamide.

1.2 Solutions for Preparing 5% Stacking Gels for SDS-Polyacrylamide Gel Electrophoresis

Solution components	Component volume (ml)	
	2 ml	5 ml
H ₂ O	1.4	3.4
30% acrylamide mix	0.33	0.83
1.0 M Tris (6.8)	0.25	0.63
10% SDS	0.02	0.05
10% ammonium persulphate	0.02	0.05
TEMED	0.002	0.005

1.3 Buffers for SDS-PAGE

SDS-gel loading buffer (3 x stock)*

150 mM Tris.HCl (pH6.8)

300 mM dithiothreitol (DTT)

6% SDS (electrophoresis grade)

0.3 % bromophenol blue

30% glycerol

*SDS-gel loading buffer is prepared as a stock solution (10 ml) without DTT. DTT will be added in to a small aliquot solution (1 ml) for daily use.

Tris-Glycine electrophoresis buffer (5 x stock)

250 mM Tris.Cl (pH 8.3)

1.25 M glycine (electrophoresis grade) (pH 8.3)

0.5 % SDS

2. Non-Denaturing Gel Electrophoresis

All the solutions are prepared in the same manner as for SDS-PAGE, except that SDS is not added into the solution.

3. Staining solution with Coomassie Brilliant Blue for Protein

Dissolve 0.25 g of Coomassie Brilliant Blue R250 in 90 ml of methanol:H₂O (1:1 v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter.

4. Destaining Solution for Coomassie Stain

30% methanol

10% acetic acid

dH₂O is added to bring volume to 100 ml.

5. Bradford Reagent for Determination of Protein Concentration

The dye reagent is prepared by dissolving 0.01% Coomassie blue G-250 in a mixture of 85% (v/v) phosphoric acid, 95% (v/v) ethanol, and water in a ratio of 10:5:85 by vol.

6. Buffers for Western blot analysis

Phosphate-buffered saline plus Tween 20 (PBS-T)

Dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.2 g of KH₂PO₄ in 800 ml of distilled H₂O. Adjust the pH to 7.4 with HCl. Add H₂O to 1 litre. 1% (v/v) of Tween 20 is added and stirred prior to use.

7. Thiosulphate solution : 10 % (w/v) sodium thiosulphate in dH₂O

Appendix IV

Methods and Solutions for Molecular Biology Work

1 Quantitation of DNA by Spectrophotometry

For quantitating the amount of DNA, reading is taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/ml for double-stranded DNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparation of DNA has OD_{260}/OD_{280} value of 1.8

2 Buffers for DNA Preparation

Alkaline Lysis Buffers for Minipreparations of Plasmid DNA

Solution I

50 mM glucose

25 mM Tris.HCl (pH 8.0)

10 mM EDTA (pH 8.0)

After the solutes are stirred until well dissolved, the solution is autoclaved for 15 min at 10 lb/sq. in. on liquid cycle, and stored at 4°C.

Solution II

0.2 N NaOH (freshly diluted from a 10 N stock)

1% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H ₂ O	28.5 ml

The resulting solution is 3 M with respect to potassium

3 Buffers for DNA Manipulation

Dilution buffer for *Sau3AI* partial digest

Buffer B	150 μ l
Acetylated BSA, 1mg/ml	150 μ l
Autoclaved distilled water to final volume	1,500 μ l

Buffer B

1 M NaCl, 100 mM Tris.HCl, 50 mM MgCl₂, 10 mM 2-mercaptoethanol,, pH 7.3

Buffer2

50 mM NaCl, 10 mM Tris.HCl, 10 mM MgCl₂, 1mM DTT, pH 7.9

Buffer 3

100 mM NaCl, 50 mM Tris.HCl, 10 mM MgCl₂, 1mM DTT, pH 7.9

Buffer4

50 mM potassium acetate, 20 mM Tris.Acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9

4 Commonly Used Buffers

Tris-EDTA (TE), (pH 8.0)

10 mM Tris.HCl (pH 8.0)

1 mM EDTA (pH 8.0)

Tris-acetate (TAE) (concentrated stock solution ,50X)

Per litre: 242 g Tris base
 57.1 ml glacial acetic acid
 100 ml 0.5 EDTA (pH 8.0)

Tris.Borate (TBE) (concentrated stock solution, 5X)

Per litre: 54 g Tris base

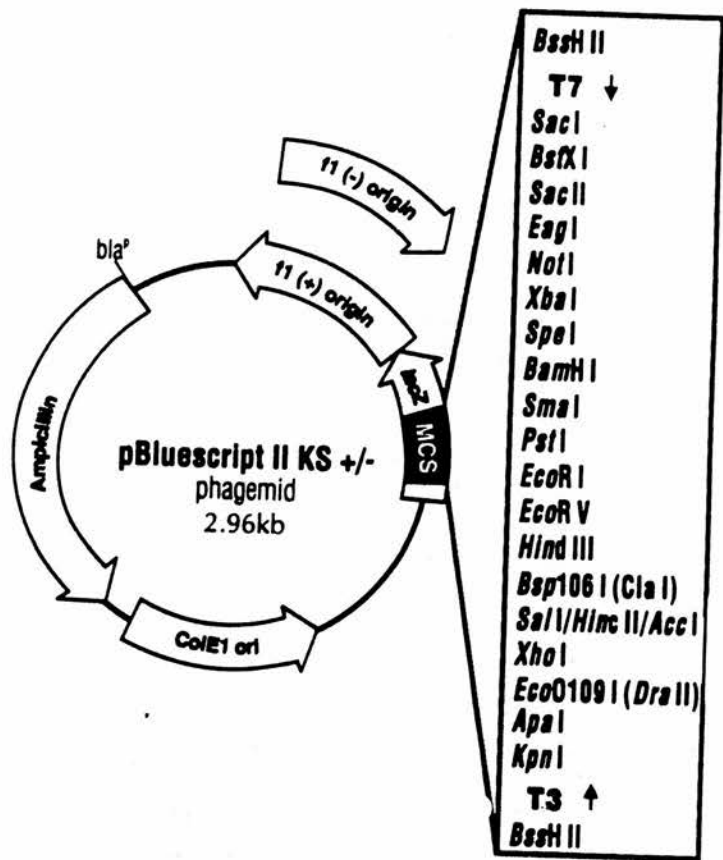
27.5 g boric acid

20 ml 0.5 M EDTA (pH 8.0)

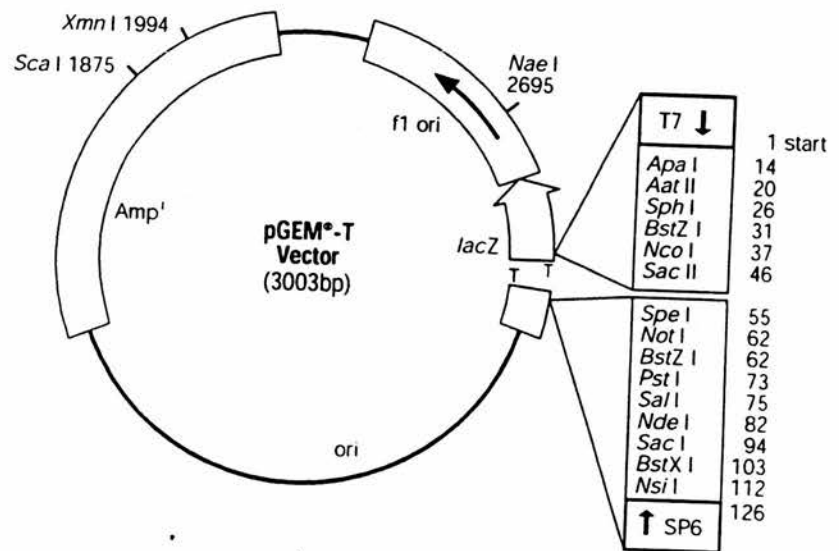
Appendix V

Plasmid Maps

1 Plasmid pBluescript II KS (-)



2 Plasmid pGEM-T



Appendix VI

Blastx Search for Chitinase Sequences

Total number of 3,841 nucleotides of P3C1/*Cla* I subclone was subjected to GenBank using Blastx search. The amino acid sequences from 110 sources that showed similarities with the P3C1/*Cla* I sequence were displayed, but only full details of the ten sequences that produce high-scoring segment pairs are given as follows:

Pir//PC4104	Chitinase A from <i>Alteromonas</i> sp. (fragment) Length = 799
Sp//P32823	Chitinase A precursor (Chi-A) from <i>Alteromonas</i> sp. Length = 820
Pir//A40633	Chitinase (Chi-85) from <i>Alteromonas</i> Length = 820
Gi/281113	Chitinase from <i>Enterobacter</i> sp. Length = 563
Pir//S60651	Chitinase precursor from <i>Serratia marcescens</i> Length = 563
Sp/P07254	Chitinase A precursor form <i>Serratia marcescens</i> Length = 563
Pdb/1CTN	Chitinase A from <i>Serratia marcescens</i> Length = 540
Gi/1899048	Chitinase Chia_Entag from <i>Enterobacter agglomerans</i> Length = 562
Gi/152818	Chitinase from <i>Serratia marcescens</i> Length = 562
Pir//A25090	Chitinase (Chi A Protein precursor, aa -23 to 583) from <i>Serratia marcescens</i> Length = 561

gi 2353792	(AF016541) beta-galactosidase alp...	+2	281	4.3e-29	1
gi 595775	(U13869) lacZ alpha peptide [Clon...	+2	276	4.5e-29	2
gi 2967701	(AF050098) chitinase [Trichoderma...	+1	98	4.9e-29	7
sp P11220 CHIT_STRPL	CHITINASE 63 PRECURSOR /pir JH05...	+1	93	8.6e-29	7
gnl PID d1010547	(D63841) modified alpha peptide o...	+2	278	1.1e-28	1
gnl PID d1010545	(D63840) modified alpha peptide o...	+2	278	1.1e-28	1
gnl PID d1006770	(D29829) beta-galactosidase alpha...	+2	278	1.1e-28	1
pir I60307	beta-galactosidase, alpha peptide...	+2	278	1.1e-28	1
gi 595780	(U13871) lacZ alpha peptide [Clon...	+2	276	2.1e-28	1
gi 567890	(L37352) beta-galactosidase-compl...	+2	270	2.4e-28	2
gnl PID d1006772	(D29830) beta-galactosidase alpha...	+2	272	3.2e-28	2
gnl PID d1006774	(D29831) beta-galactosidase alpha...	+2	274	4.0e-28	1
gi 455369	(M19265) lacZ' [Cloning vector]	+2	274	4.0e-28	1
gi 1016680	(U30830) beta-galactosidase [Clon...	+2	274	4.0e-28	1
gi 2981187	(AF049063) lacZ alpha peptide [Ex...	+2	274	4.0e-28	1
gi 2343029	(U75322) beta-galactosidase [Clon...	+2	274	4.0e-28	1
gnl PID d1010551	(D63843) modified alpha peptide o...	+2	274	4.0e-28	1
gi 1332396	(M74187) ZZ:beta-Gal' IgG-binding...	+2	274	4.0e-28	1
gnl PID e350493	(Y14836) beta-galactosidase [Phag...	+2	274	4.0e-28	1
gi 567893	(L37382) beta-galactosidase-compl...	+2	274	4.0e-28	1
gnl PID e350489	(Y14837) beta-galactosidase [Clon...	+2	274	4.0e-28	1
gnl PID e350491	(Y14835) beta-galactosidase [Phag...	+2	274	4.0e-28	1
gi 2981192	(AF049064) lacZ alpha peptide [Ex...	+2	274	4.0e-28	1
sp P00722 BGAL_ECOLI	BETA-GALACTOSIDASE (LACTASE) /gi ...	+2	273	5.4e-28	1
gi 825756	(U12391) beta-galactosidase alpha...	+2	273	5.4e-28	1
gnl PID d1014256	(D88215) alpha-peptide of beta-ga...	+2	273	5.4e-28	1
gi 455368	(M13163) lacZ' [Cloning vector]	+2	273	5.4e-28	1
gi 403937	(U01668) beta-galactosidase fragm...	+2	273	5.4e-28	1
gi 41904	(X16313) lacZ 5'-region [Escheric...	+2	273	5.4e-28	1
gnl PID e1228697	(AJ001494) beta-galactosidase [sy...	+2	273	5.4e-28	1
gi 1673616	(U76715) LacZ [Cloning vector pBR...	+2	273	5.4e-28	1
pir GBEC	beta-galactosidase (EC 3.2.1.23) ...	+2	273	5.4e-28	1
gi 1236970	(U50331) beta galactosidase alpha...	+2	273	5.4e-28	1
gi 995518	(X91197) pid:e198830 [Escherichia...	+2	273	5.4e-28	1
gnl PID d1010549	(D63842) modified alpha peptide o...	+2	272	7.4e-28	2
sp P36909 CHIT_STRLI	CHITINASE C PRECURSOR /gnl PID d1...	+3	95	1.0e-27	7
gnl PID e220269	(Z68924) Chitinase [Clostridium t...	+1	98	1.3e-27	8
gi 531829	(U12390) beta-galactosidase alpha...	+2	273	1.3e-27	2
gnl PID e263423	(Y07631) beta-D-galactosidase [un...	+2	270	1.4e-27	1
gi 434648	(U03991) beta-galactosidase alpha...	+2	270	1.4e-27	1
gi 2654021	(AF031813) maltose binding protei...	+2	270	1.4e-27	1
gi 2501755	(U66885) lacZ-alpha [bacteriophag...	+2	270	1.4e-27	1
gi 603174	(U17499) lac alpha peptide [Cloni...	+2	270	1.4e-27	1
gi 208131	(M77169) beta-galactosidase alpha...	+2	270	1.4e-27	1
gi 2623823	(AF031088) maltose binding protei...	+2	270	1.4e-27	1
gi 208984	(M88655) Modified lacZ' [Cloning ...	+2	270	1.4e-27	1
gi 312368	(X70493) lacZ [Bacteriophage M13m...	+2	270	1.4e-27	1
gi 208991	(M74186) protein A signal fusion ...	+2	270	1.4e-27	1
gi 993048	(U33186) beta-galactosidase alpha...	+2	270	1.4e-27	1
gi 987050	(X65335) lacZ gene product [unide...	+2	270	1.4e-27	1
gi 2218144	(AF004910) lac alpha peptide [Clo...	+2	270	1.4e-27	1
gi 603177	(U17500) lac alpha peptide [Cloni...	+2	270	1.4e-27	1
gi 392881	(U00800) lacZ alpha peptide [Esch...	+2	270	1.4e-27	1
gi 209095	(M21875) beta-galactosidase alpha...	+2	270	1.4e-27	1
gi 209004	(M36415) pfxblue fusion protein [...	+2	270	1.4e-27	1
gi 1658409	(U73899) beta-galactosidase [Clon...	+2	269	1.5e-27	2
gi 208243	(M34519) b-galactosidase [Cloning...	+2	269	1.9e-27	1
gi 348687	(L02937) beta-galactosidase alpha...	+2	269	1.9e-27	1
gi 1529324	(U12540) beta-galactosidase alpha...	+2	269	1.9e-27	1
gi 2071947	(U84006) beta-galactosidase [Expr...	+2	267	3.7e-27	1
gi 1005973	(U32311) beta-galactosidase alpha...	+2	265	6.9e-27	1
gi 644832	(U19930) beta-galactosidase [Clon...	+2	265	6.9e-27	1
gi 111041	(L18869) chitinase-like protein [...	+1	185	8.8e-27	3

Reference: Gish, Warren and David J. States (1993). Identification of protein coding regions by database similarity search. Nat. Genet. 3:266-72. Altschul, Stephen F., Warren Gish, Webb Miller, Eugene W. Myers, and David J. Lipman (1990). Basic local alignment search tool. J. Mol. Biol. 215:403-10.

Notice: statistical significance is estimated under the assumption that the equivalent of one entire reading frame in the query sequence codes for protein and that significant alignments will involve only coding reading frames.

Query= tmpseq_1
(3841 letters)

Translating both strands of query sequence in all 6 reading frames

Database: Non-redundant GenBank CDS
translations+PDB+SwissProt+SPupdate+PIR
299,576 sequences; 90,372,249 total letters.

Searching.....done

Sequences producing High-scoring Segment Pairs:		Reading Frame	High Score	Smallest Sum Probability P(N)	N
pir PC4106	chitinase (EC 3.2.1.14) A - Alter...	+1	832	2.8e-251	11
sp P32823 CHIA_ALTSO	CHITINASE A PRECURSOR (CHI-A) /gn...	+1	832	9.7e-251	11
pir A40633	chitinase (EC 3.2.1.14) Chi-85 - ...	+1	828	1.8e-250	11
gi 2811113	(U35121) chitinase [Enterobacter ...	+3	414	3.9e-155	10
gi 483820	(U09139) chitinase protein precur...	+3	317	5.4e-153	13
pir S60651	chitinase precursor - Serratia ma...	+3	417	1.7e-152	9
sp P07254 CHIA_SERMA	CHITINASE A PRECURSOR	+3	414	1.1e-151	9
pdb ICTN	Chitinase A (E.C.3.2.1.14) (Ph 5...	+3	414	2.0e-151	9
gi 1899048	(U59304) chitinase Chia_Entag [En...	+3	423	2.6e-148	9
gi 152818	(L01455) chitinase [Serratia marc...	+3	414	5.7e-147	9
pir A25090	chitinase (EC 3.2.1.14) - Serrati...	+3	406	3.2e-145	9
sp P41684 CHIT_NPVAC	PROBABLE ENDOCHITINASE PRECURSOR ...	+3	352	5.6e-128	9
sp O10363 CHIT_NPVOP	PROBABLE ENDOCHITINASE PRECURSOR ...	+3	329	3.5e-127	10
gi 2351556	(U72030) chitinase [Choristoneura...	+3	328	1.4e-113	12
gi 2078320	(U67265) chitinase [Helicoverpa z...	+3	333	2.0e-109	8
gi 773414	(U23751) beta galactosidase [Clon...	+2	413	5.0e-62	2
sp P20533 CHI1_BACCI	CHITINASE A1 PRECURSOR /pir A383...	+1	112	1.5e-43	8
gi 494736	(M18327) LacOPZ-alpha peptide fro...	-2	275	5.7e-43	2
gnl PID d1010477	(D63702) chitinase [Kurthia zopfii]	+3	109	6.8e-43	8
gi 458468	(U07025) chitinase [Janthinobacte...	+1	125	1.4e-38	6
pir S47133	chitinase (EC 3.2.1.14) - fungus ...	+1	99	7.2e-37	10
gi 2429326	(AF014950) chitinase A [Stenotrop...	+1	119	1.8e-36	7
gi 1845337	(U71214) chitinase [Bacillus lich...	+1	110	3.3e-36	7
gi 1132426	(U39779) beta-galactosidase alpha...	+2	278	6.4e-36	2
gnl PID d1014181	(D87894) chitinase [Rhizopus micr...	+1	91	3.7e-32	10
pir JQ1975	chitinase (EC 3.2.1.14) 1 precurs...	+3	98	5.6e-32	7
gi 2565425	(AF027497) chitinase CHIT42 [Meta...	+3	100	7.3e-32	9
sp P32470 CHI1_APHAL	CHITINASE 1 PRECURSOR /gi 429026 ...	+3	98	2.5e-31	7
pir JC2135	chitinase (EC 3.2.1.14) precursor...	+1	112	1.8e-30	7
pir JC4565	chitinase (EC 3.2.1.14) 1 - Cocci...	+3	92	3.5e-30	8
gi 1457963	(U60807) complement fixation-chit...	+3	92	3.5e-30	8
gi 1255728	(U33265) complement fixation anti...	+3	92	3.5e-30	8
gi 2738109	(U88560) endochitinase [Trichoder...	+1	100	5.9e-30	9
pir S68121	chitinase I precursor - fungus (T...	+1	101	6.0e-30	7
sp P48827 CHI4_TRIHA	42 KD ENDOCHITINASE PRECURSOR /pi...	+1	101	2.7e-29	7
gi 26464	(U26464) beta-galactosidase alpha...	+2	282	3.1e-29	2
gi 26464	(U26464) beta-galactosidase alpha...	+2	274	3.5e-29	2

Appendix VII

Literature Review

Year	Titles
1969	The chitinase of <i>Serratia marcescens</i> (Monreal and Reese, 1969)
1978	Properties of chitinase from <i>Vibrio alginolyticus</i> , as assayed with the chromogenic substrate 3,4-dinitrophenyl tetra- <i>N</i> -acetylchitotetraoside (Aribisala and Gooday, 1978)
1981	Chitinase-overproducing mutant of <i>Serratia marcescens</i> (Reid and Ogrydziak, 1981)
1985	The chitinase system of <i>Streptomyces</i> sp. ATCC 11238 and its significance for fungal cell wall degradation (Beyer and Diekmann, 1985)
1986	Chitinase determinants of <i>Vibrio vulnificus</i> : gene cloning and applications of a chitinase probe (Wortman <i>et al.</i> , 1986)
1988	Molecular cloning, expression, purification, and characterization of a chitinase from <i>Vibrio parahaemolyticus</i> (Laine <i>et al.</i> , 1988)
1989	Nucleotide sequence of the chitinase B gene of <i>Serratia marcescens</i> QMB1466 (Harper and Dunsmuir, 1989)
	Amino acid sequence of chitinase from <i>Streptomyces erythraeus</i> (Kamei <i>et al.</i> , 1989)

Chitinase studies from bacteria (continued)

1990 Gene cloning of chitinase A1 from *Bacillus circulans* WL-12 revealed its evolutionary relationship to *Serratia* chitinase and to the type III homology units of fibronectin (Watanabe *et al.*, 1990)

1992 Thermostable, salt tolerant, wide pH range novel chitobiase from *Vibrio parahaemolyticus*: isolation, characterization, molecular cloning, and expression (Zhu *et al.*, 1992)

Purification and characterization of a novel type of chitinase from *Vibrio alginolyticus* TK-22 (Murao *et al.*, 1992)

Purification, properties, and partial amino acid sequence of chitinase from a marine *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1992)

Some properties of a chitinase from a marine luminous Bacterium, *Vibrio fischeri* strain COT-A136 (Fukasawa *et al.* 1992)

Occurrence of chitin-degrading enzymes in marine and fresh water microorganisms (Ivanova *et al.*, 1992)

1993 Cloning, sequencing, and expression of a chitinase gene from a marine bacterium, *Alteromonas* sp. strain O-7 (Tsujibo *et al.*, 1993)

Purification and some properties of chitinase produced by *Vibrio* sp. (Takahashi *et al.*, 1993)

Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity (Watanabe *et al.*, 1993)

Chitinase studies from bacteria (continued)

- 1994 Cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas* sp. No. 10S-24 (Ueda *et al.*, 1994)

Crystal structure of a bacterial chitinase at 2.3 angstrom resolution (Perrakis *et al.*, 1994)

Molecular cloning and nucleotide sequence of the gene encoding chitinase II from *Aeromonas* sp. NO 10-24 (Ueda *et al.* 1994)

- 1995 Binding and substrate specificities of a *Streptomyces olivaceoviridis* chitinase in comparison with its proteolytically processed form (Blaak and Schrempf 1995)

Expression in *Escherichia coli* of a gene encoding a thermostable chitinase from *Streptomyces thermoviolaceus* OPC520 (Tsujiibo *et al.*, 1995)

Molecular cloning of *Acinetobacter* sp. WC-17 gene encoding chitinase (Shin *et al.*, 1995)

Isolation and characterization of *Acinetobacter* sp. WC-17 producing chitinase (Shin *et al.*, 1995)

Cloning and sequencing of a gene encoding the 69-kDa extracellular chitinase of *Janthinobacterium lividum* (Gleave *et al.* 1995)

Identification of the positions of disulfide bonds of chitinase from a marine bacterium, *Alteromonas* sp. strain O-7 (Hayashi *et al.*, 1995)

Enhanced production of a *Serratia marcescens* chitinase in PEG dextran aqueous 2-phase systems (Chen and Lee, 1995)

Chitinase studies from bacteria (continued)

- 1995 Cloning and sequencing of ChiC gene of *Bacillus circulans* WL-12 and relationship of its product to some other chitinases and chitinase-like proteins (Alam *et al.*, 1995)

An investigation of aquatic bacteria capable of utilizing chitin as the sole source of nutrients (Osawa and Koga, 1995)

- 1996 Three chitinase genes (chi A, chi C and chi D) comprise the chitinase system of *Bacillus circulans* WL-12 (Alam *et al.*, 1996)

Purification and properties of two chitinases from *Vibrio alginolyticus* H-8 (Ohishi *et al.*, 1996)

Structure-function studies on the chitinolytic enzymes of *Serratia marcescens* chitinase and chitobiase (Vorgias *et al.*, 1996)

Expression of chitinase-encoding genes from *Aeromonas hydrophila* and *Pseudomonas maltophilia* in *Bacillus thuringiensis* subsp. *israelensis* (Wiwat *et al.*, 1996)

The Isolation and characterization of a rumen chitinolytic bacterium (Kopečný *et al.*, 1996)

Production of chitinase and β -N-acetylglucosaminidase by internal bacteria of pinnipedian animals (Sugita *et al.*, 1996)

Production of chitinolytic enzymes from a novel species of *Aeromonas* (Huang *et al.* 1996)

Chitinase studies from bacteria (continued)

- 1996 Isolation of chitin-utilizing bacterium and production of its extracellular chitinase (Cheol-Joo *et al.*, 1996)

N-Acetylglucosaminidase (chitobiase) from *Serratia marcescens*: gene sequence, and protein production and purification in *Escherichia coli* (Tews *et al.*, 1996)

Chitinases from *Bacillus cereus*: isolation and characteristics (Trachuk *et al.*, 1996)

Bacillus ehimensis sp. nov and *Bacillus chitinolyticus* sp. nov, new chitinolytic members of the genus *Bacillus* (Kuroshima *et al.*, 1996)

A novel chitinase inhibitor from a marine bacterium, *Pseudomonas* sp. (Izumida *et al.*, 1996)

Cloning of a cluster of chitinase genes from *Aeromonas* sp. NO 10S-24 (Shiro *et al.*, 1996)

Chitinases of *Bacillus licheniformis* B-6839: Isolation and properties (Trachuk *et al.*, 1996)

Cloning, sequencing, and characterization of the *nucH* gene encoding an extracellular nuclease from *Aeromonas hydrophila* JMP636 (Dodd and Pemberton, 1996)

Comparative studies of chitinase A and chitinase B from *Serratia marcescens* (Brurberg *et al.*, 1996)

Chitinase studies from bacteria (continued)

1996 Expression of the chitinase III gene of *Aeromonas* sp. NO 10S-24 in *Escherichia coli* (Ueda *et al.*, 1996)

1997 Local and systematic induction of beta-1,3-glucanase and chitinase in coffee leaves protected against *Hemileia vastatrix* by *Bacillus thuringiensis* (Guzzo and Martins, 1997)

Purification and characterization of two bifunctional chitinases/lysozymes extracellularly produced by *Pseudomonas aeruginosa* K-187 in a shrimp and crab shell powder medium (Wang and Chang, 1997)

Properties of the chitinase of the antifungal biocontrol agent *Streptomyces lydicus* WYEC108 (Mahadevan and Crawford, 1997)

Purification and characterization of the chitinase (ChiA) from *Enterobacter* sp. G-1 (Park *et al.*, 1997)

Chitinolytic complex of *Serratia marcescens* and peculiarities of its biosynthesis (Porfireva *et al.*, 1997)

Genetic analysis of the chitinase system of *Serratia marcescens* 2170 (Watanabe *et al.*, 1997)

Cloning, sequencing, and expression of the gene encoding *Clostridium paraputrificum* chitinase ChiB and analysis of the functions of novel cadherin-like domains and a chitin-binding domain (Morimoto *et al.*, 1997)

- 1997 Isolation and characterization of the 54-kDa and 22-kDa chitinase genes of *Serratia marcescens* KCTC2172 (Gal *et al.*, 1997)

Industrial enzymes from marine microorganisms: The Indian scenario (Chandrasekaran, 1997)

Inhibition of egg hatch of the potato cyst nematode *Globodera rostochiensis* by chitinase-producing bacteria (Cronin *et al.*, 1997)

Biodegradation of chitin with enzymes and vital components (Saimoto *et al.*, 1997)

4-Methylumbelliferyl glycosides of *N*-acetyl 4-thiochito-oligosaccharides as fluorogenic substrates for chitodextrinase from *Vibrio furnissii* (Wang *et al.*, 1997)

Evolution of immunoglobulin-like modules in chitinases: their structural flexibility and functional implications (Perrakis *et al.*, 1997)

Expression and characterization of the recombination gene encoding chitinase from *Aeromonas caviae* (Lin *et al.* 1997)

Optimal dissolved oxygen concentration for the production of chitinases by *Serratia marcescens* (Khoury *et al.*, 1997)

General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae* (Sandkvist *et al.*, 1997)

Chitinolytic activity of an endophytic strain of *Bacillus cereus* (Pleban *et al.*, 1997)

Chitinase studies from bacteria (continued)

- 1997 Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans* (Chernin *et al.*, 1997)

Chitin degradation proteins produced by the marine bacterium *Vibrio harveyi* on different forms of chitin (Svitil *et al.* 1997)

Use of a promoterless *LacZ* gene insertion to investigate chitinase gene expression in the marine bacterium *Pseudoalteromonas* *sp.* strain (Techkarnjanaruk *et al.*, 1997)

Isolation and characterization of chitinase from a flake-chitin degrading marine bacterium, *Aeromonas hydrophila* H-2330 (Hiraga *et al.*, 1997)

Molecular cloning, nucleotide sequencing, and regulation of the ChiA gene encoding one of chitinase from *Enterobacter* *sp.* G-1 (Park *et al.*, 1997)

Cloning of a chitinase gene into *Bacillus thuringiensis* subsp. *aizawai* for enhanced insecticidal activity (Tantimavanich *et al.*, 1997)

Wild-type *Escherichia coli* grows on the chitin disaccharides, *N,N'*-diacetylchitobiose, by expression the *cel* operon (Keyhani and Roseman, 1997)

- 1998 Characterization of chitinase C from a marine bacterium, *Alteromonas* *sp.* strain O-7, and its corresponding gene and domain structure (Tsujibo *et al.*, 1998)

Cloning of the 52-kDa chitinase gene from *Serratia marcescens* KCTC2172 and its proteolytic cleavage into an active 35-kDa enzyme (Gal *et al.*, 1998)

1998 Anti-fungal properties of chitinolytic dune soil bacteria (Deboer *et al.*, 1998)

Chitin binding protein (CBP21) in the culture supernatant of *Serratia marcescens* 2170 (Suzuki *et al.*, 1998)

Isolation, purification and properties of a thermostable chitinase from an alkalophilic *Bacillus sp.* BG-11 (Bhushan and Hoondal, 1998)

Inhibition of two family 18 chitinases by various allosamidin derivatives (SpindlerBarth *et al.*, 1998)

Antifungal activity of chitinolytic bacteria isolated from airtight stored cereal grain (Frandsberg and Schnurer, 1998)

The role of enzyme distortion in the single displacement mechanism of family 19 chitinases (Brameld and Goddard, 1998)

Substrate distortion to a boat conformation at subsite-1 is critical in the mechanism of family 18 chitinases (Brameld and Goddard, 1998)

A chitin-binding domain in a marine bacterial chitinase and other microbial chitinases; implications for the ecology and evolution of 1,4-beta-glycanases (Svitil and Kirchman, 1998)

Multiple chitinase enzymes from a single gene *Bacillus licheniformis* TP-1 (Tantimavanich *et al.* 1998)

GlkA is involved in glucose repression of chitinase production in *Streptomyces lividans* (Saito *et al.*, 1998)

1998 Reversible immobilization of chitinase via coupling to reversible solubly soluble polymer (Wang and Chio, 1998)

High efficient expression in *Escherichia coli* of chitinase gene cloned from *Bacillus circulans* C-2 (Wang *et al.*, 1998)

Substrate assistance in the mechanism of family 18 chitinases: Theoretical studies of potential intermediates and inhibitors (Brameld *et al.*, 19998)

Molecular cloning of chitinase genes from *Vibrio anguillarum* and *V. parahaemolyticus* (Hirono *et al.*, 1998)
